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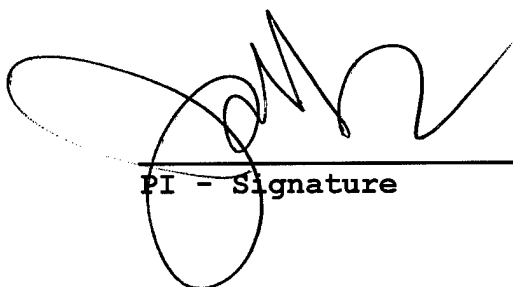
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**Annual Report**  
**DAMD17-98-1-8563**

**John C. Reed, MD, Ph.D.**

**Functions of Beta- and Gamma-Catenins in Prostate  
Cancer**

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## INTRODUCTION

The Wnt signaling pathway is conserved from humans to insects and controls a variety of aspects of cell differentiation, migration, and division (reviewed in 1). In humans, multiprotein complexes that include the tumor suppressor APC and the catenin-family proteins constitute essential components of the Wnt pathway. Catenins link the actin cytoskeleton to cell adhesion proteins, including E-cadherin and desmosomal proteins. Loss of these connections, can release  $\beta$ -catenin, allowing it to translocate to the nucleus, bind Tcf/LEF family transcription factors and induce the expression of several genes involved in cell division and oncogenesis, including *C-MYC* and *CYCLIN-D1* (2-4). APC controls the levels of  $\beta$ -catenin, collaborating with the kinase GSK3 and the F-box protein  $\beta$ -Tcrp to induce polyubiquitination and proteasome-dependent degradation of  $\beta$ -catenin. Thus, APC-assisted degradation of  $\beta$ -catenin prevents transactivation of genes that drive the cell cycle. Disruptions of the human equivalent of the Wnt signaling pathway occur commonly in human epithelial malignancies, including loss of E-cadherin expression, mutations that inactivate APC, and mutations that dysregulate catenin-family proteins (reviewed in 5). Defects in the cadherin/catenin/APC axis are often associated with cancer progression to metastatic disease (6, 7).

We have discovered that the protein Siah-1 binds to APC (8). Siah-1 is an E3 protein, which binds ubiquitin-conjugating enzymes (E2s), targeting them to various substrates in cells and thereby inducing their polyubiquitination and subsequent turnover by the proteasome (9). In contrast to the previously described mechanism for  $\beta$ -catenin degradation which depends on phosphorylation of  $\beta$ -catenin and phosphorylation-dependent interactions of  $\beta$ -catenin with the F-box protein  $\beta$ -Tcrp (10), Siah-1 can promote phosphorylation-independent,  $\beta$ -Tcrp-independent degradation of  $\beta$ -catenin (8). Thus, Siah-1 represents an alternative pathway for controlling signaling through the Wnt-pathway in human cells, and may therefore function as a tumor suppressor that collaborates with APC.

To test this hypothesis, we are generating transgenic mice that over-express in the prostate under the control of a probasin promoter either wild-type Siah-1 or a mutant of Siah-1 which cannot bind E2s and which functions as a trans-dominant inhibitor of the endogenous full-length Siah-1 protein (8, 9, 11). By studying the physiology of the prostate glands of these mice and by mating them to oncogene-harboring mice, which express SV40 Large T antigen in the prostate, we will gain critical insights into the in vivo significance of the Siah-1 pathway for  $\beta$ -catenin degradation and tumor suppression.

## **BODY**

### **OBJECTIVES**

The revised and approved objectives of our project are to:

1. Generate transgenic mice that overexpress Siah-1 or Siah-1( $\Delta$ RING) in the prostate under a probasin-promoter.
2. Examine the histoarchitecture, expression of  $\beta$ -catenin, rates of cell proliferation, and rates of apoptosis in the prostate glands of these mice under physiological conditions and after androgen deprivation.
3. Breed Siah-1 and Siah-1( $\Delta$ RING) transgenic mice with SV40-LgT/prostate mice, thus examining whether Siah modulate prostate carcinogenesis and/or metastasis in vivo.

### **PROGRESS**

**Objective #1.** Generate transgenic mice that overexpress Siah-1 or Siah-1( $\Delta$ RING) in the prostate under a probasin-promoter.

We have constructed vectors in which cDNAs encoding Siah-1 or Siah-1( $\Delta$ RING) are under the control of the probasin promoter. These have been transferred by stable transfection into a prostate cancer line to verify that they express properly. When this analysis is completed, the construct DNAs will be microinjected into mouse eggs and the transgenic lines will be generated.

In addition, we have started preparations for generating a SIP gene knockout mouse. SIP is a novel protein that we cloned due to its ability to interact with Siah-1. SIP connects Siah-1 to Skp, a central component of the SCF-complexes which control ubiquitination and turnover of many proteins involved in cell division, including  $\beta$ -catenin. A targeted insertion of the SIP gene was found during a search of the Lexigen, Inc. database, permitting us to order ES cells which contain a targeted insertion of this gene and thus begin our attempts to generate a SIP knockout mouse. These mice will be critical for understanding the significance of SIP for regulation of Siah-1-mediated degradation of  $\beta$ -catenin in vivo.

**Objective #2.** Examine the histoarchitecture,  $\beta$ -catenin levels, rates of cell proliferation, and rates of apoptosis in the prostate glands of these mice under physiological conditions and after castration.

Not applicable until animals are generated.

**Objective #3.** Breed Siah-1 and Siah-1( $\Delta$ RING) transgenic mice with SV40-LgT/prostate mice, thus examining whether Siah modulates prostate carcinogenesis and/or metastasis in vivo.

A colony of probasin-LgT transgenic mice has been established.

#### **PUBLICATIONS ATTRIBUTED TO THIS GRANT PERIOD**

The studies in which we describe the original observations linking Siah to APC have been submitted for publication. Copies are provided in the appendix section.

1. Liu J., Matsuzawa S., Reed J.C., White R., Matsunami N.: Siah-1 binds adenomatous polyposis coli protein and regulates  $\beta$ -catenin turnover. Submitted, 1999.
2. Matsuzawa S, Reed J.C.: SIP: A protein that bridges Siah-1 to SCF-complexes involved in protein ubiquitination and degradation. Submitted, 1999

#### **CONCLUSION**

$\beta$ -catenin serves as a critical relayer of signals from cell-cell adhesion events at the submembranous cytoskeleton to the nucleus, where it controls the expression of genes involved in cell cycle. The factors that control the levels of  $\beta$ -catenin in epithelial cells are complex. We have discovered novel proteins involved in  $\beta$ -catenin degradation. Gaining an improved understanding of the mechanisms relevant to the control of  $\beta$ -catenin in vivo is essential for devising strategies for restoring normal growth control to epithelial malignancies. The work ongoing in this proposal will establish the in vivo significance of novel  $\beta$ -catenin regulating proteins in the prostate using transgenic mouse technology.



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11. Matsuzawa, S. and Reed, J. SIP: A protein that bridges siah-1 to scf-complexes involved in protein ubiquitination and degradation, Submitted, 1999.

# **Siah-1 Binds Adenomatous Polyposis Coli Protein and Regulates $\beta$ -Catenin Turnover**

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## ABSTRACT

The adenomatous polyposis coli (*APC*) tumor suppressor protein acts as a cytoplasmic scaffold to form a Wnt-regulated signaling complex that mediates the phosphorylation-dependent degradation of  $\beta$ -catenin. We have now found that the human homologue of *Drosophila seven in absentia*, Siah-1, also interacts with APC. Expression of Siah-1 promoted the post-translational down-regulation of  $\beta$ -catenin and inhibited Tcf/Lef-dependent transcription. Unexpectedly, mutant  $\beta$ -catenin which can not be phosphorylated by GSK3 $\beta$  also showed a Siah-1-dependent decrease. This decrease in  $\beta$ -catenin level was not blocked by a dominant-negative  $\beta$ -TrCP. These results indicate that APC and Siah-1 mediate a novel pathway that can down-regulate  $\beta$ -catenin.

## INTRODUCTION

Inherited tumor predispositions provide an opportunity to define critical early genetic events in the development of tumors. An inherited colon cancer predisposition, familial adenomatous polyposis, is caused by mutant alleles of the adenomatous polyposis coli (*APC*) gene (Grodin et al 1991, Joslyn et al. 1991, Kinzler et al. 1991, Nishisho et al 1991). The early appearance of hundreds or thousands of colon polyps in this inherited disorder indicates that mutations in the *APC* gene can be rate-limiting in polyp development. The majority of sporadic colon polyps and carcinomas also carry mutated *APC* genes, establishing that somatic mutations in the *APC* gene are an early event in the development of colon cancer (Powell et al. 1992). Several lines of evidence indicate that the tumor suppressor activity of the *APC* protein relies, at least in

part, on its ability to bind and subsequently down-regulate the cytoplasmic levels of  $\beta$ -catenin (Munemitsu et al. 1995, Rubinfeld et al. 1997, Korinek et al. 1997, Morin et al. 1997).  $\beta$ -Catenin is a multifunctional protein that plays an essential role in the transduction of Wnt signaling and is also a component of the cadherin cell adhesion complex (Cadinan & Nusse 1997). In the absence of Wnt signaling, the cytoplasmic level of  $\beta$ -catenin is kept low through interaction with an APC, axin, protein phosphatase 2A, and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) complex, where  $\beta$ -catenin may be phosphorylated by GSK3 $\beta$  (Behrens J et al. 1998, Hart et al. 1998, Ikeda et al. 1998, Seeling et al 1999). This phosphorylation event results in the ubiquitin-mediated proteasomal degradation of  $\beta$ -catenin (Aberle et al 1997). Recent studies have shown that degradation of phosphorylated  $\beta$ -catenin is triggered by specific interaction with the F-box/WD40-repeat protein,  $\beta$ -TrCP (FWD1/Slimb) (Jiang 1998, Winston et al. 1999, Spencer 1999, Kitagawa et al. 1999, Liu et al 1999, Hart et al. 1999, Latres et al. 1999, Fuchs et al. 1999).  $\beta$ -TrCP thus serves as an intracellular receptor for phosphorylated  $\beta$ -catenin, forming a Skp1/Cullin/F-box protein <sup>$\beta$ -TrCP</sup> (SCF <sup>$\beta$ -TrCP</sup>) ubiquitin ligase complex that ubiquitinates  $\beta$ -catenin, leading to its proteasomal degradation. During normal development, activation of Wnt signaling leads to the inactivation of GSK3 $\beta$ , which results in accumulation of cytoplasmic  $\beta$ -catenin (Peifer 1997). At elevated cytoplasmic levels,  $\beta$ -catenin translocates to the nucleus and cooperates with a member of the T-cell factor (Tcf)/lymphocyte enhancer-binding factor (Lef) family of high mobility group box transcription factors to activate expression of target genes, such as c-Myc (He et al. 1998) and cyclin D1 (Tetsu et al. 1999, Shtutman et al. 1999). Support for the suggestion of  $\beta$ -catenin as the etiologic target of APC in carcinogenesis is provided by the observation of

point mutations in  $\beta$ -catenin that alter putative GSK3 $\beta$  phosphorylation residues were reported in melanomas (Rubinfeld et al. 1997), colon cancers (Morin et al. 1997, Sparks et al. 1998), prostate cancers (Voeller et al. 1998), hepatic cancers (de La Coste et al. 1999), skin cancers (Chan et al. 1999) and experimentally induced cancers (Dashwood et al. 1998). These mutations would weaken or abolish the association of  $\beta$ -catenin with  $\beta$ -TrCP, leading to accumulation of cytoplasmic  $\beta$ -catenin.

Although both GSK3 $\beta$  and  $\beta$ -TrCP can participate in the APC-mediated down-regulation of cytoplasmic  $\beta$ -catenin, it is possible that other pathways might also be important. The existence of such a pathway has been postulated recently (Easwan et al. 1999). Here, we report that the human homologue of the *Drosophila seven in absentia*, Siah-1, which is a mediator of p53-dependent cell-cycle arrest and apoptosis (Matsuzawa et al. 1998, Roperch et al. 1999), binds the carboxyl-terminus of APC protein and post-translationally down-regulates  $\beta$ -catenin through a mechanism independent of both GSK3 $\beta$ -mediated phosphorylation and  $\beta$ -TrCP-mediated proteasome pathway.

## RESULTS

### Identification interaction between of APC and Siah-1

Yeast two-hybrid cDNA library screening identified the interaction between the carboxyl-terminal 155 amino acids of APC and Siah-1, a human homologue of the *Drosophila seven in absentia* (Sina) gene. In *Drosophila*, Sina gene is required for formation of the R7 photoreceptor cells during eye development (Carthew et al. 1990, Li et al. 1997, Tang et al. 1997). To confirm the interaction between APC and Siah-1, we

tested a number of known constructs in a directed two-hybrid assay for interaction with the carboxyl-terminal 155 amino acids of APC. As shown in Table 1, the interaction of Siah-1 with APC requires the carboxyl-terminal 194-298 amino acid sequence of Siah-1. APC also interacted with Siah-2, which has high amino acid sequence homology with Siah-1 (data not shown) (Hu et al. 1997b). There was no detectable interaction with B AG-1, Bax, Ras, or Fas (Table 1).

To confirm the results of the yeast two-hybrid assay, several Siah-1 and APC constructs were tested by a GST pull-down assay. *In vitro* translated <sup>35</sup>S-labeled Siah-1 (full-length, aa 1-298), ΔN-Siah-1 (the amino-terminal deletion, leaving a peptide with aa 97-298) and ΔC-Siah-1 (the carboxyl-terminal deletion, which leaves a peptide carrying aa 1-193) (Figure 1a, b) were tested for binding to various GST-APC carboxyl-terminal fusion proteins (Figure 2). As in the yeast two-hybrid system, Siah-1 and ΔN-Siah-1, but not ΔC-Siah-1, associated with APC, indicating that the carboxyl-terminal sequences of Siah-1 are required for binding to APC. No interaction was observed with GST alone.

### **Siah-1 down-regulates Tcf/Lef reporter activity**

Both Sina and Siah proteins have been implicated in the proteasomal degradation of proteins with which they interact (Li et al. 1997, Tang et al. 1997, Hu et al. 1997a, Zhang et al. 1998). However, our initial experiments showed that expression of Siah-1 had no dramatic effect on the abundance of APC protein (data not shown). Since one essential function of APC is to act as a cytoplasmic scaffold for the assembly of molecules in the Wnt signaling pathway, the interaction of Siah-1 with APC suggested that Siah-1 might regulate Wnt signaling. To test this hypothesis, we assessed whether

increased expression of Siah-1 would have any effect on the Tcf/Lef reporter activity (Korinek et al. 1997) in 293T cells. As shown in Figure 3a, expression of Siah-1 dramatically decreased Tcf/Lef reporter activity in a dose dependent manner. On the other hand, expression of the amino-terminal deletion mutant  $\Delta$ N-Siah-1 increased Tcf/Lef reporter activity (Figure 3b). The  $\Delta$ N-Siah-1 protein is known to function as a trans-dominant inhibitor of endogenous Siah-1 (Hu & Fearon 1999)

### **Siah-1A down-regulates $\beta$ -catenin**

Since the RING domain at the amino-terminus of Siah has been shown to trigger protein degradation (Hu & Fearon 1999), we hypothesized that Siah-1 might promote degradation of  $\beta$ -catenin or other positive regulators of Wnt signaling. To address this question, we ectopically expressed Myc-tagged  $\beta$ -catenin in 293T cells with either Siah-1 or  $\Delta$ N-Siah-1. As shown in Figure 4a, expression of Siah-1 led to a decrease in the amount of Myc- $\beta$ -catenin. On the other hand,  $\Delta$ N-Siah-1 increased the amount of Myc- $\beta$ -catenin. During these experiments, the amount of other proteins such as c-Myc and I $\kappa$ B remained unchanged (data not shown). The effect of Siah-1 on  $\beta$ -catenin was also apparent from pulse-chase experiments in which Myc-tagged  $\beta$ -catenin was co-expressed with Siah-1 or  $\Delta$ N-Siah-1 in 293T cells and the rate of Myc- $\beta$ -catenin turnover was examined (Figure 4b). The half-life of Myc- $\beta$ -catenin was reduced to approximately 40 minutes when co-expressed with Siah-1 compared with a half-life of several hours when co-expressed with vector control. On the contrary, the half-life of Myc- $\beta$ -catenin was increased when co-expressed with  $\Delta$ N-Siah-1. These results demonstrate that Siah-1 promotes down-regulation of  $\beta$ -catenin in a post-translational manner and  $\Delta$ N-Siah-1 stabilizes  $\beta$ -catenin.

### **Siah-1 down-regulates mutant $\beta$ -catenin which has substitutions in GSK3 $\beta$ phosphorylation residues**

To determine whether Siah-1 regulates the abundance of  $\beta$ -catenin through a mechanism that requires GSK3 $\beta$ -mediated phosphorylation, we first examined the effect of Siah-1 on mutant FLAG-tagged  $\beta$ -catenin which has substitutions in putative GSK3 $\beta$  phosphorylation residues (serine and threonine amino acid residues between 33 and 45). Such mutations would produce stable  $\beta$ -catenin protein that accumulates in the cell (Aberle et al. 1997, Orford et al. 1997, Rubinfeld et al. 1997, Kitagawa et al. 1999). Consistent with these observations, overexpression of GSK3 $\beta$  led to a decrease in the amount of wild-type FLAG- $\beta$ -catenin, but it had no significant effect on the amount of mutant FLAG- $\beta$ -catenin (Figure 5a). However, as shown in Figure 5b, expression of Siah-1 led to a decrease in the amount of mutant FLAG- $\beta$ -catenin as well as wild-type FLAG- $\beta$ -catenin. A similar result was obtained with an amino-terminal truncation mutant  $\Delta$ N131- $\beta$ -catenin (Barth et al. 1997) (data not shown). These results indicate that Siah-1 mediates down-regulation of  $\beta$ -catenin independently of phosphorylation of the amino-terminal serines and threonines by GSK3 $\beta$ .

### **Dominant-negative $\beta$ -TrCP does not block Siah-1-mediated down-regulation of $\beta$ -catenin**

Recent studies have shown that the F-box/WD40-repeat protein,  $\beta$ -TrCP, serves as an intracellular receptor for phosphorylated  $\beta$ -catenin and subsequently forms the SCF <sup>$\beta$ -TrCP</sup> ubiquitin ligase complex to process ubiquitination and degradation of  $\beta$ -catenin (Aberle 1997, Jiang 1998, Winston et al. 1999, Spencer 1999, Kitagawa et al. 1999, Hart et al. 1999, Latres et al. 1999, Liu et al. 1999, Fuchs et al. 1999). Since our



results indicate that Siah-1 promotes degradation of  $\beta$ -catenin through a mechanism independent of GSK3 $\beta$  phosphorylation, it seemed possible that the degradation of  $\beta$ -catenin mediated by Siah-1 might occur by a mechanism distinct from the  $\beta$ -TrCP pathway. To test this hypothesis, we blocked the  $\beta$ -TrCP pathway by transfecting 293T cells with a plasmid expressing the dominant-negative form of  $\beta$ -TrCP (F-box deletion mutant). This dominant-negative  $\beta$ -TrCP has been shown to bind phosphorylated  $\beta$ -catenin, but is unable to form a SCF <sup>$\beta$ -TrCP</sup> ubiquitin ligase complex, resulting consequently in accumulation of cytoplasmic  $\beta$ -catenin (Hart et al. 1999, Latres 1999). As shown in Figure 6, expression of dominant-negative  $\beta$ -TrCP increased the amount of Myc- $\beta$ -catenin. Co-expression of Siah-1 with dominant-negative  $\beta$ -TrCP, however, dramatically decreased the amount of Myc- $\beta$ -catenin. Taken together, the results presented here indicate that Siah-1 promotes the down-regulation of  $\beta$ -catenin in a mechanism independent of both GSK3 $\beta$ -mediated phosphorylation and the  $\beta$ -TrCP-mediated proteasome pathway.

## DISCUSSION

In this report, we show that Siah-1, the human homologue of the *Drosophila seven in absentia*, binds the carboxyl-terminus of APC protein. Expression of Siah-1 reduced the abundance of  $\beta$ -catenin protein and inhibited Tcf/Lef-dependent transcription in mammalian cells. Pulse-chase analysis indicates that Siah-1 promotes down-regulation of  $\beta$ -catenin in a post-translational manner. Overexpression of either non-phosphorylatable mutant of  $\beta$ -catenin or a dominant-negative  $\beta$ -TrCP failed to block Siah-1-mediated down-regulation of  $\beta$ -catenin. These results indicate that Siah-1

mediates degradation of  $\beta$ -catenin through a novel mechanism, independent of both GSK3 $\beta$ -mediated phosphorylation and  $\beta$ -TrCP-mediated proteasome pathway. At present, we do not know how Siah-1 promotes degradation of  $\beta$ -catenin. Preliminary observations indicate that inclusion of a proteasome inhibitor, MG-132, blocks Siah-1-induced down-regulation of  $\beta$ -catenin in the presence of dominant-negative  $\beta$ -TrCP, suggesting that an alternative pathway for polyubiquitination of  $\beta$ -catenin is involved. Such pathway might include other members of kinases and F-box proteins. Indeed, Easwaran *et al.* (1999) have recently shown that a serine kinase other than GSK3 $\beta$  modulates APC-mediated down-regulation of  $\beta$ -catenin. However, because Siah-1 reportedly binds ubiquitin conjugating enzymes (E2) directly *via* its RING domain (Matsuzawa & Reed, submitted), it is possible that Siah-1 functions in collaboration with APC as an E3 complex which catalyzes transfer of ubiquitin from E2 enzymes to  $\beta$ -catenin without requirements for other proteins.

Previous studies had revealed that there are multiple binding sites for  $\beta$ -catenin exist on APC, including three consensus 15-amino acid repeat sequences (Su *et al.* 1993) and centrally located 20-amino acid repeat sequences (Rubinfeld *et al.* 1995). Although Siah-1 binds the carboxyl-terminus of APC, it is still unclear whether the presence of APC is a prerequisite for Siah-1 to down-regulate  $\beta$ -catenin. However, expression of Siah-1 in the colon cancer cell lines SW480 and DLD1, which express only truncated APC (Smith *et al.* 1993), failed to decrease Tcf/Lef reporter activity (J.L., R.W. and N.M. unpublished data). This observation suggests that down-regulation of  $\beta$ -catenin by Siah-1 may require intact APC protein.

Siah is the mammalian homologue of the *Drosophila seven in absentia* (Sina), required for formation of the R7 photoreceptor cells during eye development of the fly. Two highly conserved human homologues (hSiah-1 and hSiah-2) and three murine homologues (mSiah-1A, mSiah-1B, and mSiah-2) have been described (Della et al. 1993, Holloway et al. 1997, Hu et al. 1997b). Both Sina and Siah have been implicated in the proteolysis of certain proteins with which they interact. In *Drosophila*, Sina promotes ubiquitin-mediated, proteasomal degradation of Tramtrack, a transcriptional repressor of R7 photoreceptor cell fate (Li et al. 1997, Tang et al. 1997). In mammalian cells, Sina and Siah proteins bind to the cytoplasmic domain of the DCC (deleted in colon cancer) protein and promote its proteolysis via the proteasome pathway (Hu et al. 1997a). Also in mammalian cells, mSiah-2 targets the ubiquitin-mediated proteasomal degradation of N-CoR (nuclear receptor co-repressor) protein, which has been shown to form a repressor complex with nuclear receptor, Sin3 and histone deacetylase (Zhang et al. 1998). hSiah-2 binds and inhibits the product of the Vav oncogene, however this inhibitory effect seems independent of proteasomal degradation (Germani et al. 1999). Although the overall physiological significance of Siah proteins in mammalian cells remains unclear, recent studies suggest that Siah may be involved in cell-cycle regulation, apoptosis, and tumorigenesis. mSiah-1B was identified among a group of genes induced during p53-induced apoptosis and G1-arrest in murine M1 myeloid leukemia cells (Amson et al. 1996). mSiah-1B was also strongly induced in murine fibroblasts undergoing apoptotic cell death (Hu et al. 1997a). hSiah-1 was identified as a p53-p21<sup>WAF1</sup> inducible gene activated in its expression during physiological apoptosis and various *in vitro* model systems of tumor suppression

(Nemani et al. 1996, Linares-Cruz et al 1998) and ectopic expression of Siah-1 can induce apoptosis and promote tumor suppression (Roperch et al. 1999). Recently, Matsuzawa et al. (1998) have shown that Siah-1 is indeed a mediator of p53-dependent cell-cycle arrest and is directly inhibited by the Hsc70-binding protein BAG-1. However, the mechanism by which Siah proteins regulate cell-cycle, apoptosis, and tumorigenesis is still unknown. As mutations in APC and  $\beta$ -catenin genes had been implicated in multiple malignancies and APC is involved in cell-cycle regulation (Grodén et al. 1995) and apoptosis (Morin et al. 1996), our finding would provide new insights into the functions of the Siah-1 protein.

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### **METHODS**

#### **Two-hybrid assays and library screening**

Library screening by the yeast two-hybrid method was performed as described (Matsuzawa et al. 1998) using the pGilda plasmid encoding human Siah-1 (22-298) as a bait, a human Jurkat T-cell cDNA library (gift from Brian Seed), and the EGY48 strain *S.cerevisiae* (MATa, trp1, ura3, his, leu2::plexApo6-leu2). Cells were grown in either YPD medium with 1% yeast extract, 2% polypeptone, and 2% glucose, or in Burkholder's minimal medium (BMM) fortified with appropriate amino-acids as described previously (Sato et al. 1994). Transformations were performed by a LiCl method using 0.25 µg of pJG4-5-cDNA library DNA, and 5 µg of denatured salmon sperm carrier DNA. Clones that formed on Leu-deficient BMM plates containing 2% galactose/ 1% raffinose were transferred to BMM plates containing leucine and 2% glucose, and filter assays were performed for β-galactosidase measurements as previously described (Hanada et al. 1995, Sato et al. 1994). From an initial screen of ~2.0 X 10<sup>7</sup> transformants, 322 clones were identified that trans-activated the LEU2 reporter gene based on ability to grow on leucine-deficient media. Of those, 32 colonies were also positive for β-galactosidase. These 32 candidate transformants were then cured of the LexA/BAG-1 bait plasmid by growth in media containing histidine and then mated with each of 5 different indicator strains of cells containing one of following LexA bait proteins: Siah-1(22-298), Bax (1-171), v-Ras, Fas (191-335), or BAG-1. The mating strain was RFY206 (MATα, his3Δ200, leu2-3, lys2Δ201, ura3-52, trp1Δ::hisG), which had been transformed with pGilda-Siah-1 or various control proteins and selected on histidine-deficient media. This resulted in 11 clones which displayed specific two-hybrid interactions with Siah-1. DNA sequencing analysis revealed 3 cDNAs encoding portions of APC(2681-2843).

## Plasmids

Plasmids (pcDNA, Clontech) expressing Siah-1,  $\Delta$ N-Siah-1 and  $\Delta$ C-Siah-1 were described previously (Matsuzawa et al. 1998). For production of GST-APC fusion constructs, DNA cassettes encoding carboxyl-terminal APC were PCR amplified and inserted in frame into downstream of GST gene in pGEX-2TK (Pharmacia) using standard PCR cloning techniques. The primers used to amplify APC (aa 2688-2843) were APC2688 (5'-GCGCGC GGATCC ATGGAA AAGGCA AATCCA AACATT-3') and RAPC (5'-GGCCGG GGATCC TTAAAC AGATGT CACAAG GTAAGA-3'). The primers for APC (aa 2543-2843) were APC2543 (5'-GCGCGC GGATCC ATGTCA GGAACC TGGAAA CGTGAG-3') and RAPC. A plasmid expressing Myc-tagged  $\beta$ -catenin was kindly provided by Dr. P. Polakis. For generating plasmids encoding Flag-tagged  $\beta$ -catenins, primers KPNFBCAT (5'-GCCAGT GGTACC GCCGCC ACCATG GATTAC AAGGAT GACGAC GATAAG GCTACT CAAGCT GATTTG-3'), containing the Kozak sequence and FLAG-tag, and XBABCAT (5'-ACAGCT ATGACC TCTAGA TTACAG GTCAGT ATCAAA CCAGG-3') were first used to PCR amplify the wild-type  $\beta$ -catenin. To construct Flag-tagged mutant  $\beta$ -catenin, which has substitutions in putative GSK3 $\beta$  phosphorylation residues (serine and threonine amino acid residues between 33 and 45), DNA fragments encoding the amino- and the carboxyl-terminal regions were separately PCR amplified with primer sets, KPNFBCAT and MutTACB (5'-AAAAGG AGCTGT GGCAGT GGCACC AAAATG GATTCC AAAGTC CAGGTA AGACTG TTG-3'), and XBABCAT and MutBCAT (5'-TTTGGA ATCCAT TTTGGT GCCACT GCCACA GCTCCT TTTCTG AGTGGT AAAGGC AAT-3'), respectively. The two PCR products were mixed and subjected to the 2<sup>nd</sup> PCR

reaction to obtain the full-length mutant  $\beta$ -catenin. Both wild-type and mutant  $\beta$ -catenin DNA fragments were digested with KpnI and BamHI, and cloned into expression vector pcDNA. For dominant-negative  $\beta$ -TrCP, wild-type  $\beta$ -TrCP cDNA was first amplified by RT-PCR from mRNA of colon cancer cell line SW480 using primers F1 (5'-GCGCGC GGATCC GCCGCC ACCATG GACTAC AAGGAC GACGAT GACAAG GACCCG GCCGAG GCGGTG CTG-3'), containing the Kozak sequence and FLAG-tag, and R1 (5'-GGCCGG TCTAGA TTATCT GGAGAT GTAGGT GTATGT-3'). The dominant-negative  $\beta$ -TrCP, which does not have F-box, was constructed as follows. The amino-terminal region of  $\beta$ -TrCP (aa 1-147) was amplified with primers F1 and R2 (5'-GGCCGG CTCGAG AGCAGT TATGAA ATCTCT CTG-3'), and was digested with BamH I and XhoI. The carboxyl-terminal region of  $\beta$ -TrCP (aa 193-569) was amplified with primers F2 (5'-GCGCGC CTCGAG AGAATG GTCAGG ACAGAT-3') and R1, and was digested with XhoI and XbaI. The two fragments were ligated into BamHI/XbaI sites of pcDNA. All constructs were confirmed by DNA sequencing.

#### ***In vitro* binding assays**

APC cDNAs encoding the carboxyl terminus (aa 2688-2843 and aa 2543-2843) were expressed in DH5 $\alpha$  cells (Life Technologies), and affinity-purified using glutathione-Sepharose. Purified GST fusion proteins (10  $\mu$ g) and 5  $\mu$ l of rabbit reticulocyte lysates (TNT coupled reticulocyte lysate system; Promega) containing  $^{35}$ S-labeled, *in vitro* translated (IVT) Siah-1 proteins were incubated in 100  $\mu$ l of buffer containing 25mM Tris (ph7.5), 50mM NaCl, 1mM dithiothreitol, 0.1%NP-40 and 0.1mg/ml bovine serum albumin at 4°C for 2 hours. GST-fusion proteins were recovered on glutathione-Sepharose beads. The beads were washed four times with L-buffer (PBS, 0.1% NP-40

and 0.1% Triton X-100) and boiled in Laemmli-SDS sample buffer. The eluted proteins were subjected to SDS-PAGE and the dried gel was analyzed with a PhosphorImager (Molecular Dynamics).

### **Cell culture and transfections**

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. To determine Tcf/Lef reporter activity, subconfluent 293T cells were co-transfected with a reporter construct (0.5 µg of pTOPFLASH or pFOPFLASH) (Korinek et al. 1997, Morin et al. 1997), an internal control (0.05 µg of pCMVβ-gal) and indicated plasmids in six-well plates by calcium phosphate method (Chen & Okayama 1987). The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Luciferase and β-galactosidase activity was measured 24 hours after transfection. Tcf/Lef reporter activity was determined as described (Korinek et al. 1997, Morin et al. 1997). To detect Myc-β-catenin, FLAG-β-catenin and GFP proteins, 293T cells were lysed directly in Laemmli-SDS sample buffer 48 hours after transfection. Total cellular proteins (40 µg /lane) were separated by 4-20 % gradient Tris-glycine SDS-PAGE and were transferred to nitrocellulose membrane. Proteins were detected with primary antibodies and horseradish peroxidase-conjugated secondary antibodies using an ECL system (Amersham). Primary antibodies used were mouse anti-c-Myc monoclonal antibody (9E10, Santa Cruz Biotechnology), mouse anti-FLAG monoclonal antibody (M2, Sigma), and mouse anti-GFP monoclonal antibody (Clontech). Blots were analyzed with a Lumi-Imager (Boehringer Mannheim).



### **Pulse-chase analysis**

To perform pulse-chase analysis of ectopically expressed Myc-tagged  $\beta$ -catenin, 239T cells were transiently transfected in six-well plates. 36 hours after transfection, cells were pulse-labeled for one hour with 0.1 mCi of  $^{35}\text{S}$ -methionine and cysteine per each well, and then chased with cold media. Cells were lysed in RIPA buffer (0.05 M Tris buffer, pH7.2, 0.15M NaCl, 1% Triton-X100, 1% deoxycholate, 0.1%SDS) supplemented with protease inhibitors at the indicated times. After centrifugation of the lysates, Myc- $\beta$ -catenin was immunoprecipitated from supernatants by mouse anti-c-Myc monoclonal antibody (9E10) conjugated to agarose (Santa Cruz Biotechnology). After three washes with RIPA buffer, immunoprecipitates were subjected to SDS-PAGE. Dried gels were analyzed with a PhosphorImager (Molecular Dynamics).

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## FIGURE LEGENDS

Figure 1 Schematic representation of APC and Siah-1 proteins and deletion constructs used in this study. **a**, APC is a 2843-amino-acid protein (Grodén et al. 1991, Joslyn et al. 1991, Kinzler et al. 1991, Nishisho et al. 1991) that contains Armadillo repeats in the amino-terminus (Peifer et al. 1994), 15- and 20-aa repeats in the central region (Rubinfeld et al. 1993, Su et al. 1993, Hulsken et al. 1994, Rubinfeld et al. 1995), and a basic region in the carboxyl-terminus (Grodén et al. 1991, Joslyn et al. 1991, Kinzler et al. 1991, Nishisho et al. 1991). The carboxyl-terminus also contains a TXV sequence, which mediates DLG binding (Matsumine et al. 1996). **b**, Siah-1 protein (Matsuzawa et al. 1998). Shown is the position of the conserved RING-finger domain.

Figure 2 ***In vitro* binding of Siah-1 to APC.** The indicated Siah-1 constructs were expressed as <sup>35</sup>S-labeled proteins by *in vitro* transcription and translation and then incubated with the indicated GST-APC fusion proteins. GST-fusion proteins were recovered on glutathione-agarose beads and subjected to SDS-PAGE. The dried gel was analyzed with a PhosphorImager (Molecular Dynamics). The IVT (*in vitro* translated) samples represent 40 % of that used in the binding analysis.

Figure 3 **Effect of Siah-1 on Tcf/Lef reporter activity.** 293T cells were co-transfected with a reporter construct (pTOPFLASH or pFOPFLASH Korinek et al. 1997, Morin et al. 1997), an internal control (pCMV $\beta$ -gal) and indicated plasmids. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Luciferase and  $\beta$ -galactosidase activity was measured 24 hours after

transfection. Tcf/Lef reporter activity was determined as described (Korinek et al. 1997, Morin et al. 1997). The histograms are presented as the average  $\pm$  SD from duplicate experiments. **a**, Siah-1 down-regulates Tcf/Lef-reporter activity in a dose dependent manner. **b**,  $\Delta$ N-Siah-1 up-regulates Tcf/Lef-reporter activity acting as a dominant-negative form.

**Figure 4 Effect of Siah-1 on  $\beta$ -catenin levels and turnover.** **a**, 293T cells were transiently co-transfected with an internal control (pEGFP) and the indicated plasmids. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Whole cell lysates were subjected to Western analysis. Blots were probed with either a mouse monoclonal antibody to Myc epitope tag (upper panel) or a mouse monoclonal antibody to GFP (lower panel) which served as a control for transfection efficiency. The histogram shows the relative amount of Myc- $\beta$ -catenin which was corrected for transfection efficiency by using the amount of control GFP and is presented as the average  $\pm$  SD from duplicate experiment. **b**, Pulse-chase analysis of ectopically expressed Myc-tagged  $\beta$ -catenin. 239T cells were transiently co-transfected with the indicated plasmids, pulse-labeled with  $^{35}$ S-methionine and cysteine, and then chased with cold media. Cells were lysed at the indicated times and the expressed Myc- $\beta$ -catenin was recovered by immunoprecipitation via a Myc epitope tag. Immunoprecipitated Myc- $\beta$ -catenin was subjected to SDS-PAGE and dried gels were analyzed with a PhosphorImager (Molecular Dynamics). Plot of the pulse-chase analysis was presented as the average  $\pm$  SD from duplicate experiments.

**Figure 5 Siah-1 down-regulates  $\beta$ -catenin through a mechanism independent of GSK3 $\beta$ -mediated phosphorylation.** 293T cells were co-transfected with an internal control (pEGFP) and indicated plasmids. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Whole cell lysates were subjected to Western analysis. Blots were probed with either a monoclonal antibodies to FLAG epitope tag or a mouse monoclonal antibody to GFP. The histograms show the relative amount of FLAG-tagged  $\beta$ -catenin, which was corrected for transfection efficiency by using the amount of control GFP and are presented as the average  $\pm$  SD from duplicate experiments. **a**, Overexpression of GSK-3 $\beta$  down-regulates wild-type  $\beta$ -catenin but not mutant  $\beta$ -catenin which has substitutions on GSK3 $\beta$  phosphorylation residues. **b**, Siah-1 can down-regulate both wild-type and mutant  $\beta$ -catenin. WT; wild-type, Mut; mutant.

**Figure 6 Siah-1 down-regulates  $\beta$ -catenin through a mechanism independent of  $\beta$ -TrCP-mediated proteasome pathway.** Dominant-negative (DN)  $\beta$ -TrCP does not block Siah-1-mediated down-regulation of  $\beta$ -catenin. 293T cells were co-transfected with an internal control (pEGFP) and indicated plasmids. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Whole cell lysates were subjected to Western analysis. Blots were probed with either a monoclonal antibodies to Myc epitope tag or a mouse monoclonal antibody to GFP. The histograms show the relative amount of Myc-tagged  $\beta$ -catenin, which was corrected for transfection efficiency by using the amount of control GFP and are presented as the average  $\pm$  SD from duplicate experiments.

**Table 1.** Specific interaction of APC with Siah-1

LexA	B42	LEU	$\beta$ -Gal
Siah-1 (22-298)	APC (C-term. 155a.a.)	+	+
BAG-1	APC (C-term. 155a.a.)	-	-
Bax	APC (C-term. 155a.a.)	-	-
Ras	APC (C-term. 155a.a.)	-	-
Fas	APC (C-term. 155a.a.)	-	-
APC (C-term. 155a.a.)	Siah-1 (22-298)	+	+
APC (C-term. 155a.a.)	Siah-1 (22-193)	-	-
APC (C-term. 155a.a.)	Siah-1 (46-102)	-	-
APC (C-term. 155a.a.)	Siah-1 (97-298)	+	+

Plasmids producing LexA DNA-binding domain fusion proteins (1 $\mu$ g each) (listed at left) were co-transformed with 1 $\mu$ g of pJG4-5 plasmid producing B42 transactivation domain fusion proteins (listed at right) into EGY48 strain yeast. Transformed cells were grown on semi-solid media lacking leucine, or containing leucine as a control which results in equivalent amounts of growth for all transformants (data not shown). Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+).  $\beta$ -galactosidase activity of each colony was tested by filter assay and scored as blue (+) or white (-) after 60 minutes.

**Figure 1**

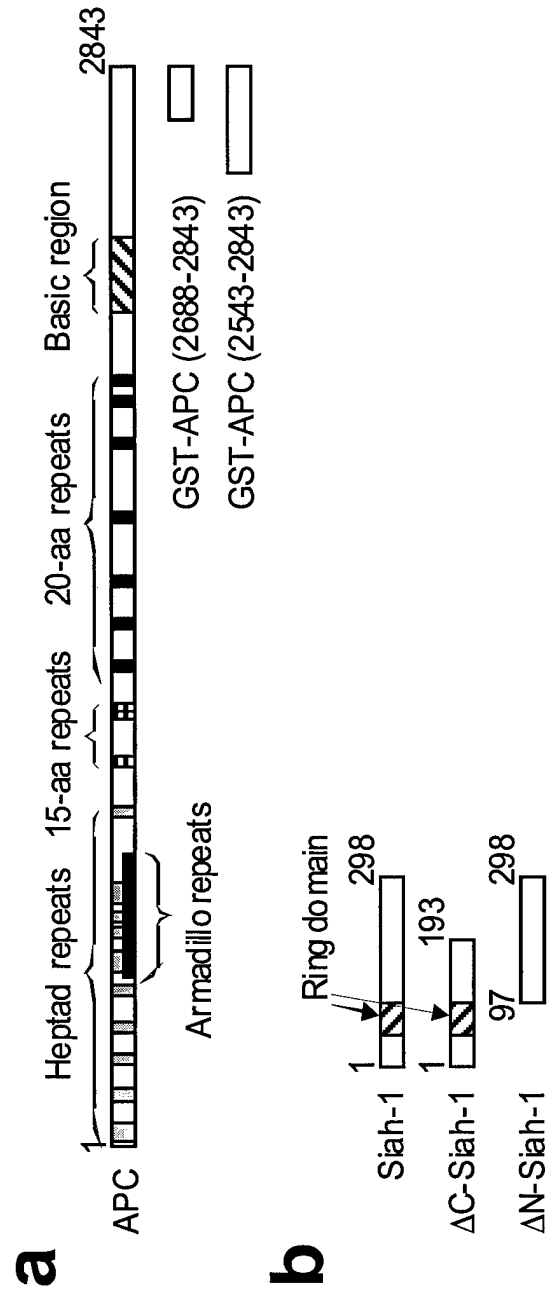




Figure 2

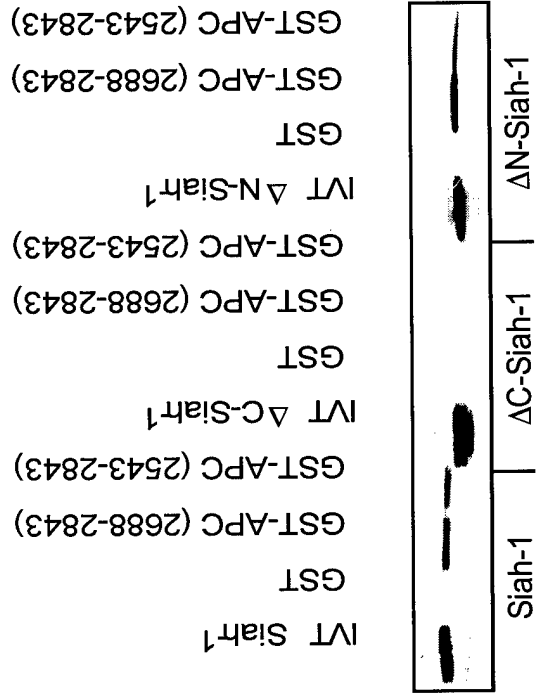
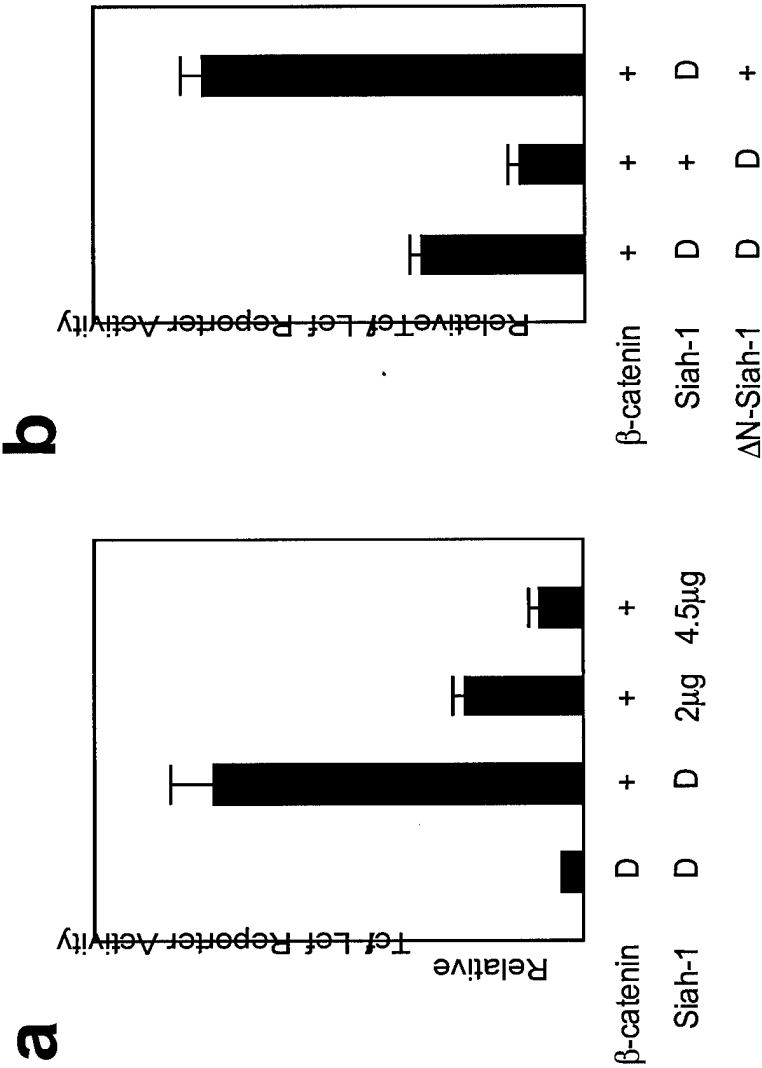
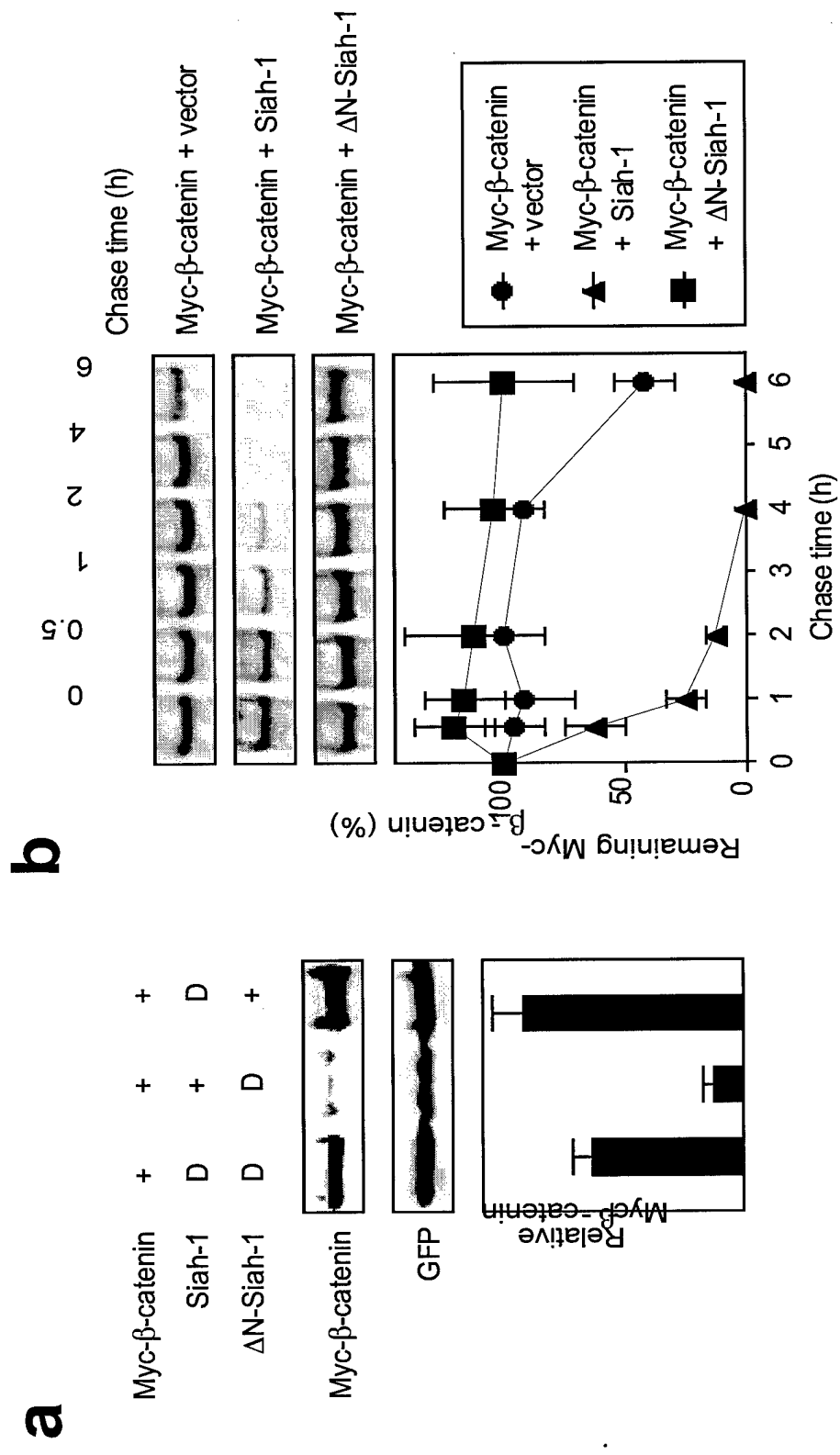


Figure 3

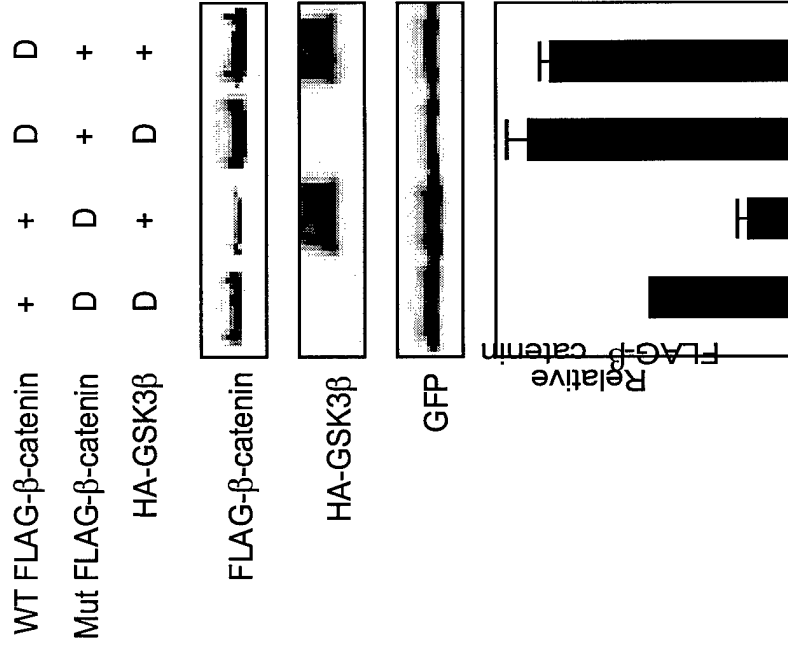


# Figure 4

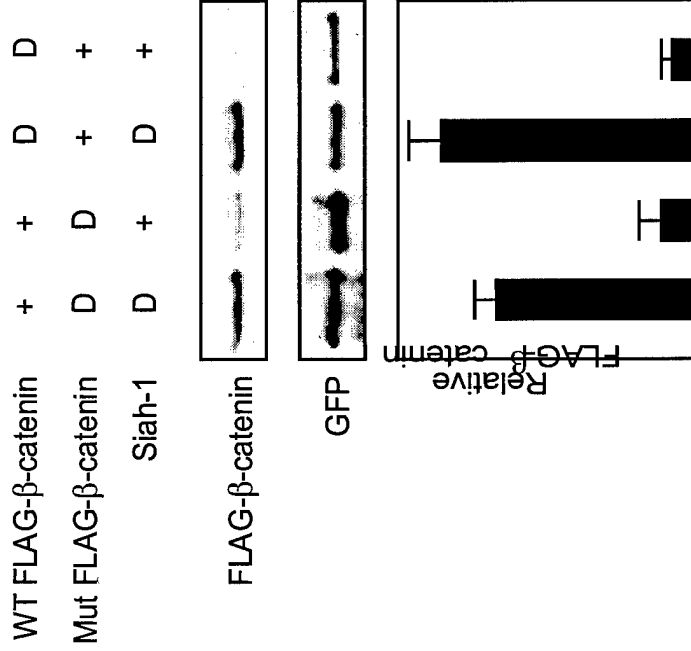


# Figure 5

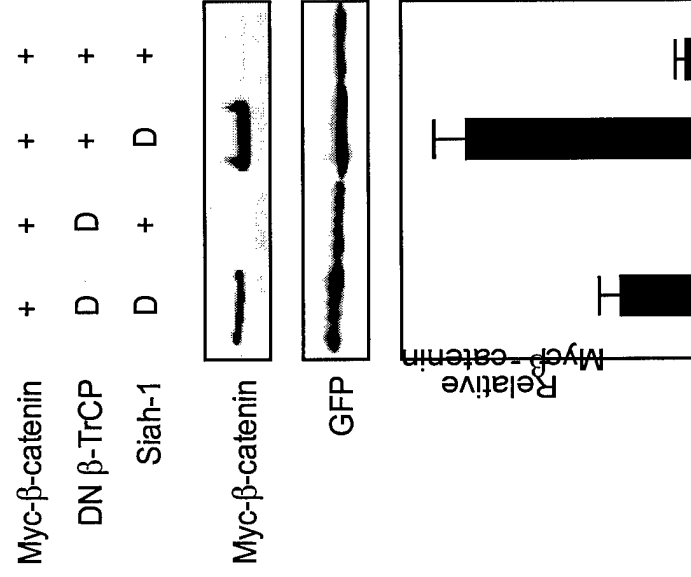
**a**



**b**



# Figure 6



**SIP: A Protein That Bridges Siah-1 To SCF-Complexes  
Involved in Protein Ubiquitination and Degradation.**

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Running Title: SIP bridges Siah-1 to Skp1.

## SUMMARY.

Specificity in targeting of proteins for degradation by polyubiquitination is controlled through protein interactions, involving E3 protein complexes which bring specific target-proteins and E2 ubiquitin-conjugating enzymes into close proximity. A protein, SIP, was identified that links two major E3-systems for targeted protein ubiquitination: (a) Sina/Siah-1 family proteins, originally implicated in R7 cell development in *Drosophila*, and (b) Skp1-Cullin-F-box (SCF) complexes, which control the temporal degradation of several cell cycle-regulatory proteins. SIP binds simultaneously to Siah-1 and Skp1 through separate domains, bridging Siah-1 to SCF complexes. Siah-1 regulates the degradation of a known SCF-substrate and induces cell cycle arrest, in a SIP-dependent manner. The findings provide a molecular explanation for genetic studies suggesting functional connections between the Sina and SCF pathways.

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## INTRODUCTION

The temporal coordination of sequential steps within the eukaryotic cell cycle is governed in large part by protein degradation, involving targeted ubiquitination of specific cell cycle regulatory proteins followed by their destruction by the proteasome reviewed in (1). Among the cell cycle regulators whose levels are controlled by ubiquitination and subsequent proteasome-dependent degradation are the cyclins (cyclins A, B, C, D1, E) and several of the cyclin-dependent kinase (cdk) inhibitory proteins including p21<sup>Waf1</sup> and p27<sup>Kip1</sup>. Defects in this highly regulated process of protein turnover have been documented in many types of cancer.

The steps involved in polyubiquitination of specific proteins in cells involves the concerted actions of E1, E2, and E3-type enzymes. E1 proteins form thioester bounds in which the sulfhydryl group of internal cysteine residues binds the carboxyl amino acid of ubiquitin, thereby activating ubiquitin for subsequent transfer to E2-family proteins. E2 family proteins then transfer activated ubiquitin to the free amino-groups of lysine side chains in target proteins directly. More often, however, E2-family proteins collaborate with E3 proteins which bind particular target proteins and orchestrate their interactions with E2s, coordinating the polyubiquitination of these target proteins in highly regulated manners (1).



E3 ubiquitin ligase functions are sometimes embodied in multiprotein complexes rather than mediated by a single protein.

SCF complexes play a critical role in the ubiquitination and degradation of a variety of cyclins, cyclin-dependent kinases (cdks), and cdk-inhibitors during the cell cycle. These multiprotein complexes function as ubiquitin ligases, and contain the Skp1 protein, at least one Cullin-family protein, and at least one F-box protein, thus the acronym SCF: Skp = S; C = Cullin; F = F-box) reviewed in (2, 3). F-box proteins contain a conserved motif, the F-box, which mediates their interactions with Skp1. The F-box proteins also contain other domains which allow them to simultaneously bind specific substrate proteins, which are then targeted for degradation via polyubiquitination, typically in a phosphorylation-dependent manner. One such F-box protein identified in humans is  $\beta$ -TRCP, which forms a SCF complex with Skp1 and Cul-1, and which interacts with  $\beta$ -catenin, targeting it for degradation (4-7).

Siah-family proteins represent mammalian homologs of the *Drosophila* Sina protein. Sina is required for R7 photoreceptor cell differentiation within the sevenless pathway (8). Sina binds a ubiquitin-conjugating enzyme (E2) via an N-terminal RING domain. Heterocomplexes of Sina and another protein called Phyllopod form a E3-

complex which interacts with a transcriptional repressor called Tramtrack, targeting it for polyubiquitination and proteosome-mediated degradation (9, 10). The destruction of Tramtrack is necessary for differentiation of R7 cells in the developing eyes of flies.

Humans contain two genes that encode Sina-like proteins, called *SIAH1* and *SIAH2* (11). Like their *Drosophila* counterpart, the Siah-1 and Siah-2 proteins bind ubiquitin conjugating enzymes (UBCs) via an N-terminal RING domain and target other proteins for degradation. Thus far, the only reported targets of Siah-mediated degradation are DCC (12) a putative tumor suppressor protein encoded by a gene which is commonly disrupted in colon cancers (13) and Nco-R, co-repressor of nuclear hormone receptors (14). Further connections between Siah-family proteins and tumor suppressor genes have been found for p53. The Siah-1 gene of mice was found among a group of immediate-early genes induced by p53 using a hemopoietic cell line as a model for p53-induced cell cycle arrest and apoptosis (15). Expression of Siah-1 was also indirectly correlated with increased apoptosis in tumor xenograph experiments, suggesting that Siah-1 could function as a tumor suppressor in some contexts (16). Recently, we have provided evidence that Siah-1 over-expression can induce cell cycle arrest independently of apoptosis in

epithelial cancer cells (17). These and other data have implicated Siah-1 in a p53-inducible pathway for cell cycle arrest which runs parallel to the well-studied p21-Waf1 pathway (17). At present, the diversity of functions of Siah-family proteins remain unclear.

Recently, genetic analysis of pathways involved in R7 cells development in *Drosophila* has suggested a functional link between Sina and SCF-complex E3 ubiquitin-ligases. Specifically, a new modulator of pathways controlling Tramtrack degradation, *ebi*, was reported to encode a protein structurally similar to the certain F-box proteins previously identified in humans, yeast, and *C. elegans* (18). However, the molecular mechanisms by which functional connection are achieved between Sina/Siah-family proteins and components of the SCF-complex has been unclear. In this study, we have identified a Siah-Interacting Protein (SIP) involved in Siah-mediated protein degradation. We show that SIP binds simultaneously to Siah1 and to Skp1, a central component of the SCF complex, thus bridging Siah1 to this E3 ubiquitin-ligase and revealing a previously unrecognized mechanism for association of Siah-family proteins with components of SCF-complexes.

## RESULTS

**Identification of human SIP as a Siah-1 binding protein.** To identify potential targets of Siah-1, we performed yeast two-hybrid screens of cDNA libraries using the human Siah-1 protein as a bait. From a pool of 11 candidate clones, 2 were found to encode Siah-1, thus reconfirming previous observations that Siah-1 forms homo-dimers or -oligomers (17, 19). Among the other cDNA clones identified by two-hybrid screening, 6 corresponded to overlapping clones, presumably derived from the same gene. These cDNAs encode polypeptides of 228 (three clones), 222 (one clone), 217 (one clone) and 80 (one clone) amino-acids length, which all share identity in the N-terminal region but which differ with respect to the length of their C-terminal portions. The longest cDNAs cloned during two-hybrid screening encompass the complete open reading frame (ORF) of a predicted 228 amino-acid protein, based on DNA sequence analysis of additional cDNAs containing portions of the 5'-untranslated region, revealing a stop codon upstream and in-frame with this ORF which is initiated by an AUG within a favorable Kozak context. A search of the PAC database revealed the complete nucleotide sequence of the corresponding gene (PAC 102G20; chromosome 1q24-25), permitting deduction of the exon-intron organization of this human gene by

comparison with these longest cDNAs (Figure 1A). DNA sequence analysis of the shortest cDNA derived by two-hybrid screening revealed an 8 amino-acid difference in the deduced ORF, followed by a stop codon, thus resulting in a predicted 80 amino-acid protein which arises through alternative mRNA splicing (Figure 1A, B). We have termed the 228 and 80 amino acid proteins encoded by these cDNAs Siah-1 Interacting Protein (SIP) and SIP-short (SIP-S), respectively. As shown in Figure 1, the shorter SIP-S protein is generated as a result of use of an upstream splice donor site within exon 2 within the *SIP* gene. The predicted human SIP protein shares 93% identity in amino acid sequence with a mouse protein, previously identified as a calcyclin-binding protein (20), suggesting that this mouse protein represents the mouse ortholog of SIP. Interestingly, hSIP was also found to have substantial amino-acid sequence similarity to Sgt-1, a protein recently shown to bind SCF-complexes in yeast (7). The human homolog of Sgt-1 is 27% identical (45% similar) to hSIP, based on amino-acid sequence comparisons (Figure 1B) and also shares 22% and 25% sequence identity (37% and 43% similarity) with the Sgt-1 proteins of *S. cerevisiae* and *S. pombe*, respectively.

**Specific interaction of SIP and SIP-S with Siah-1.** The interaction of Siah-1 with SIP and SIP-S was confirmed to be specific in two-hybrid

assays in which the recovered plasmids were re-transformed into yeast and tested against a variety of irrelevant proteins (Table 1). Interestingly, SIP also appeared to interact with itself, suggesting that it can homodimerize or -oligomerize.

To further confirm the interaction of SIP with Siah-1, *in vitro* binding assays were performed using *in vitro* translated  $^{35}\text{S}$ -labeled Siah-1 and affinity-purified GST-SIP fusion protein. ( $^{35}\text{S}$ )-Siah-1 bound to GST-SIP, whereas little or no binding to GST-CD40 (Figure 2A) and several other GST-control proteins, including GST-Bax and GST-BAG-1 (data not shown), was observed.

To examine whether the interaction of SIP with Siah-1 occurs in cells, we performed co-immunoprecipitation experiments. Since the RING domain of Siah-1 results in rapid turnover of this protein (19), we employed a Siah-1 truncation mutant lacking the N-terminal first 96 residues, Siah-1 (97-298). Accordingly, an expression plasmid encoding HA epitope-tagged Siah-1 (97-298) was transfected into 293T cells alone or in combination with plasmids encoding either myc-epitope-tagged SIP or as a negative control, HA-nm23. The resulting cell lysates were immunoprecipitated using a monoclonal antibody specific for the HA epitope (3F10) and associated myc-SIP protein was detected by

immunoblotting using an anti-myc monoclonal antibody (9E10). As shown in Figure 2B, myc-SIP co-immunoprecipitated with HA-Siah-1 whereas HA-nm23 did not. Use of control mouse IgG for immunoprecipitations instead of anti-HA further confirmed the specificity of these results (Figure 2B).

To explore the regions of Siah-1 responsible for interaction with SIP, we prepared a series of Siah-1 deletion mutants that were expressed in yeast as fusion proteins with a LexA DNA-binding domain (Figure 2C). Immunoblotting using an anti-LexA antiserum confirm production of all Siah-1 proteins at comparable levels (not shown). A N-terminal deletion mutant of Siah-1 (amino acids 97-298) that removed the RING-finger domain retained SIP-binding activity. In contrast, a C-terminal deletion mutant of Siah (amino acids 1-193) failed to interact with SIP. Thus, the N-terminal RING domain is not required for SIP binding, but the C-terminal last 106 amino acids of this protein does contain a SIP-binding domain. Similar results were obtained by in vitro binding assays, using GST-SIP fusion protein for binding to in vitro translated full-length Siah-1, Siah-1 ( $\Delta$ RING), or Siah-1 ( $\Delta$ C) proteins (not shown).

Though the Siah-1 ( $\Delta$ C) protein failed to interact with SIP in either yeast two-hybrid assay or in vitro binding experiments, this truncated

protein retained the ability to associate with Siah-1 (Figure 2C). Thus, the SIP-binding domain of Siah-1 appears to be separable from its self-association domain. Removal of the N-terminal 96 amino-acids from Siah-1 where the RING domain resides did not interfere with self-association, indicating that the RING domain is not required for this property of Siah-1. Absence of the RING domain however did abolish two-hybrid interactions of Siah-1 with the Ubiquitin-Conjugating-H5 (Ubc-H5) protein and with Ubc-H9. The RING domain of Siah was also sufficient for binding Ubc-H5 and Ubc-H9, consistent with prior investigations of the *Drosophila* Siah homolog, Sina, which interacts with the fly Ubc-H5 homolog via its RING domain (9, 10).

Next we examined the regions in SIP that are required for binding to Siah-1 and for homo-dimerization or oligomerization with itself, again using yeast two-hybrid assays (Figure 2D). From the initial two-hybrid cloning experiments, we deduced that both the SIP and SIP-S proteins contain an N-terminal domain sufficient for Siah-1 binding. In contrast, a N-terminal deletion mutant of SIP (amino acids 81-228) lacking this conserved region failed to bind Siah, though it did retain the ability to interact with full-length SIP (Figure 2D). These results imply that the region of SIP corresponding to amino-acids 1-80 is involved in Siah-1-



binding, whereas downstream regions of the protein allow self-association of SIP molecules. Interestingly, the SIP-S protein displayed no ability to bind to SIP or to itself (Figure 2D), further confirming that the self-association domain within SIP lies downstream of the Siah-binding domain.

From these experiments, we conclude that both Siah-1 and SIP are modular proteins which possess separable domains responsible for self-association and interactions with each other. The SIP-S protein, in contrast, has the ability to bind Siah-1 but lacks the domain necessary for self-association.

These experiments also suggested that Siah-1 could bridge Ubc proteins to SIP, using its RING domain to bind Ubcs and its C-terminal region (97-298) to bind SIP. To explore this possibility, yeast three-hybrid experiments were performed in which SIP was expressed as a fusion protein with a LexA DNA-binding domain and Ubc-H5 was expressed with a B42 transactivation domain, in the presence or absence of Siah-1. When co-expressed without Siah-1, LexA-SIP and B42-Ubc-H5 failed to constitute the two-hybrid interactions required for activating the reporter plasmids that allow these yeast to grow on selective media (Figure 2E). In contrast, when yeast were additionally transformed with a plasmid encoding Siah-1, reporter gene activation was induced, indicating that

Siah-1 provided the necessary bridge for bringing SIP and Ubc-H5 into close proximity. Conversely, a plasmid encoding Siah-1 ( $\Delta$ RING), which fails to bind Ubcs, did not support reporter gene activation (Figure 2E), even though the Siah-1( $\Delta$ RING) protein was produced (not shown).

**Siah-1-induced cell cycle arrest is inhibited by SIP-S.** Previously we reported that over-expression of Siah-1 inhibits cellular proliferation (17). To explore the functional consequences of SIP/Siah interactions, we examined the effects of the SIP and SIP-S proteins on Siah-1-induced cell cycle arrest in 293T epithelial cancer cells (Figure 3). For these experiments, 293T cells were transiently transfected with plasmids encoding Siah-1 alone or in combination with SIP or SIP-S. Inclusion of pEGFP plasmid encoding a Green Fluorescent Protein (GFP) served as a marker for identification of successfully transfected cells, verifying > 90% transfection efficiency. The number of viable cells was then counted at 24h post-transfection. Consistent with our previous observations (17), over-expression of Siah-1 resulted in decreased cell numbers in cultures of transfected cells after 24h, without an increase in cell death. Thus, Siah-1 suppresses proliferation of 293T cells. Co-transfection of SIP with Siah-1 did not substantially alter Siah-1-mediated growth suppression. In

contrast, the SIP-S protein partially abrogated the growth suppressive effects of Siah-1. Immunoblot analysis confirmed that the expected ~32 kDa FLAG-Siah-1, ~30 kDa myc-SIP, and ~17 kDa myc-SIP-S proteins were produced at readily detectable levels in transiently transfected 293T cells (Figure 3B). Interestingly, however, co-expression of Siah-1 with SIP resulted in less accumulation of SIP protein compared to transfections lacking Siah-1 (Figure 3B), suggesting that Siah-1 may promote turnover of SIP protein, analogous to its reported effects on other proteins it binds (12, 14)

To further explore the effects of SIP-S on cell growth, we employed a model of p53-induced cell cycle arrest wherein MCF7 breast cancer cells are UV-irradiated using a relatively low dose of radiation (10 Joules/m<sup>2</sup>), thus inducing accumulation of endogenous p53 protein and cell cycle arrest without accompanying apoptosis (17). For these experiments, MCF7 breast cancer cells were stably transfected with pcDNA3 control plasmid or plasmids encoding myc-SIP or myc-SIP-S proteins. Immunoblot analysis using anti-myc epitope antibody confirmed expression of the myc-SIP and myc-SIP-S proteins in the corresponding transfectants but not the control cells (Figure 3C). As shown, UV-irradiation of control or SIP over-expressing MCF7 cells resulted in reduced cell proliferation (Figure 3D). In contrast, over-expression of the SIP-S protein almost completely prevented

UV-induced arrest of cell cycle arrest, restoring cell division to roughly the same levels of control non-irradiated cells. We conclude therefore that SIP-S interferes with cell cycle arrest induced by over-expression of Siah-1, as well as growth inhibition by UV-irradiation in a context in which p53 has been shown to induce Siah-1 expression (17).

**SIP interacts with Skp1 of the SCF complex.** Using yeast two-hybrid cDNA library screening methods, we searched for SIP-binding proteins. From a screen of 15 million initial transformants, 16 were identified that interacted specifically with SIP. These included: (a) 2 clones encoding Siah-1, thus re-confirming our observations that SIP and Siah-1 interact; (b) 11 encoding SIP, thus confirming the ability of SIP to self-associate; and (c) 3 encoding the full-length Skp-1 protein, a cell cycle regulator and SCF component. The Skp1 protein exhibited specific interactions with the SIP in two-hybrid assays (Table 2). In contrast, the SIP-S protein did not interact with Skp1. As expected (3), Skp1 also interacted with the Cullin-family protein Cul1 and the F-box protein  $\beta$ -Trcp, whereas Siah-1 and SIP did not (Table 2). Interaction of Skp1 with SIP was confirmed by in vitro binding experiments using in vitro translated  $^{35}\text{S}$ -labeled Skp1 and affinity-

purified GST-SIP (Figure 4A), and by co-immunoprecipitation experiments involving transfected 293T cells (Figure 4B).

To map the domains in SIP required for binding Skp1 and to compare them with those involved in Siah-1-binding, truncation mutants of SIP were constructed and assayed for interactions with Siah-1 or Skp1 by two-hybrid assays. Whereas the N-terminal 61 amino acids of SIP were sufficient for binding to Siah-1, the C-terminal region of SIP was required for interactions with Skp1 (Figure 4C). Similarly, while the SIP-S protein was capable of binding to Siah-1, it failed to interact with Skp1 in either co-immunoprecipitation (Figure 4B) or yeast two-hybrid (Figure 4C) assays. Thus, different regions of SIP mediated its interactions with Siah-1 and Skp1. Similarly, truncation mutants of Skp1 were tested in two-hybrid assays, demonstrating that N-terminal residues 1-90 of Skp1 are sufficient for interactions with SIP (Figure 4D). Deletion of the first 90 amino-acids of Skp1 abolished its ability to interact with SIP in both co-immunoprecipitation (Figure 4B) and yeast two-hybrid (Figure 4D) assays. In contrast to SIP, Siah-1 failed to interact with Skp1 in two-hybrid assays.

Based on these domain mapping studies, we speculated that SIP could bridge Siah-1 to Skp1, using its N-terminal domain to bind Siah-1 and its C-terminal region for interactions with Skp1. To examine this

hypothesis, three-hybrid assays were performed using plasmids encoding Siah-1 fused to the DNA-binding domain of LexA, Skp1 fused to the transactivation domain of B42, and non-fused SIP or SIP-S proteins. As shown, expression of LexA-Siah-1 and B42-Skp1 in the absence of SIP failed to activate the LexA-responsive reporter gene encoding a *LEU2* selectable marker that permits growth of transformants on leucine-deficient media (Figure 4E). In contrast, when SIP was co-expressed with Siah-1 and Skp1 fusion proteins, a two-hybrid reaction was achieved, resulting in growth of transformants on leucine-deficient plates. Unlike SIP, the SIP-S protein which is incapable of binding Skp1 (Table 2) failed to mediate interactions between Siah-1 and Skp1 (Figure 4E). We conclude therefore that SIP can function as a molecular bridge that permits interaction of Siah-1 with Skp1, a component of SCF complexes.

#### **Siah-1 regulates degradation of $\beta$ -catenin, a known SCF-substrate.**

Skp1 is an invariable and central component of SCF complexes, which target proteins for polyubiquitination based on the specificity of particular F-box proteins (3). The F-box protein  $\beta$ -Trcp can form complexes with Skp1 and binds the target protein  $\beta$ -catenin in a phosphorylation-

dependent manner, thus promoting its SCF-mediated polyubiquitination and subsequent degradation (4-7). We utilized  $\beta$ -catenin degradation therefore as a model for assessing the functional impact of Siah-1 and SIP on an SCF-complex-mediated process in cells. Accordingly, 293T cells were transiently transfected with plasmids encoding myc-tagged  $\beta$ -catenin alone or in combination with plasmids producing Siah-1, dominant-negative Siah-1 ( $\Delta$ RING), SIP, or SIP-S. The steady-state levels of transgene-derived  $\beta$ -catenin were then monitored by immunoblotting.

Over-expression of Siah-1 resulted in a marked (> 90%) decrease in  $\beta$ -catenin protein levels (Figure 5). The residual  $\beta$ -catenin protein in lysates derived from Siah-1 over-expressing cells often migrated in SDS-PAGE as a ladder of bands or higher molecular weight smear, typical of ubiquitinated proteins. Moreover, inclusion of a proteasome inhibitor (MG132) in the culture medium prevented Siah-1-induced degradation of  $\beta$ -catenin (Figure 5), confirming a proteasome-dependent degradation process. In contrast to Siah-1, expression of the Siah-1 ( $\Delta$ RING) protein in 293T cells did not result in decreases in the levels of myc- $\beta$ -catenin protein, consistent with the inability of this protein to bind Ubcs. Re-probing the same immunoblot with an antibody recognizing GFP (included in all transfections) verified loading of equivalent amounts of protein lysates

and demonstrated specificity of the Siah-1-mediated degradation of  $\beta$ -catenin, since over-expression of Siah-1 had no effect on GFP protein levels. We conclude therefore that Siah-1 but not Siah-1 ( $\Delta$ RING) increases the efficiency of degradation of a known SCF-substrate,  $\beta$ -catenin.

In similar co-transfection experiments, over-expression of SIP protein by itself did not alter  $\beta$ -catenin levels (not shown). However, the combination of SIP and Siah-1 resulted in more potent depletion of  $\beta$ -catenin from cells compared to Siah-1 alone, such that essentially no detectable  $\beta$ -catenin remained (Figure 5). Conversely, co-expressing either SIP-S or SIP( $\Delta$ N) with Siah-1 completely nullified the effects of Siah-1 on  $\beta$ -catenin degradation, restoring  $\beta$ -catenin to control levels (Figure 5). Immunoblotting confirmed production of the SIP, SIP-S and SIP( $\Delta$ N) proteins in these experiments (Figure 5). We deduce from these experiments that Siah-1-induced degradation  $\beta$ -catenin is SIP-dependent, since the SIP-S and SIP( $\Delta$ N) proteins blocks this effect of Siah-1. The SIP-dependence of this effect is particularly supported by the observation that both SIP-S and SIP( $\Delta$ N) interfere with Siah-1-induced degradation of  $\beta$ -catenin, since SIP-S binds Siah-1 but not the SCF component Skp1 and conversely SIP( $\Delta$ N) binds Skp1 but not Siah-1.



## DISCUSSION

We have identified SIP as a novel link between Sina/Siah-family proteins and the SCF-complex component Skp1. *Sina* was first discovered in the context of genetic analysis of R7 photoreceptor cell differentiation in the developing eye of fruit flies (8). The Sina protein binds Ubc-D1, the *Drosophila* homolog of human Ubc-H5, via its RING domain and requires interactions with Phyllopodia for targeting Tramtrak for ubiquitination and degradation (9, 10, 19). Thus, Sina brings E2s to Phyllopod/Tramtrak complexes, resulting in Tramtrak ubiquitination and turnover. Recently, another gene, *ebi*, has been implicated in R7 cell development. Loss of function mutations of *ebi* prevent R7 cell differentiation and this is correlated with accumulation of Tramtrak, as determined by immunostaining (18). The *ebi* gene of *Drosophila* encodes an F-box protein with similarity to Cdc4 (yeast), Sel-10 (*C. elegans*), and Slimb (*Drosophila*), suggesting that it provides a functional connection between a Sina-regulated pathway and SCF-complexes. How this linkage between Sina and SCF-complexes is achieved in a biochemical sense however has been unclear. Our finding that SIP functions as a molecular bridge between the human homologs of Sina and the SCF-component Skp1 provides the first biochemical evidence of a physical linkage between these ubiquitin-ligase

systems, thus corroborating the genetic evidence from *Drosophila* that these two major pathways for targeted protein degradation interact.

SIP is a modular protein that contains separate domains capable of independently binding Siah-1 and Skp1. Expression of fragments of SIP which bind either Siah-1 (e.g. SIP-S) or Skp1 (e.g. SIP( $\Delta$ N)), but which are incapable of bridging these two proteins, resulted in interference with Siah-1-induced degradation of  $\beta$ -catenin and prevented Siah-1-mediated cell cycle arrest. Thus, SIP provides a functional and physical bridge between Siah-1 and Skp1, and this bridging role for SIP appears to functionally connect Siah-1 to a pathway that controls  $\beta$ -catenin degradation and cell proliferation. The SIP-S protein (a dominant-inhibitory isoform of SIP produced by alternative mRNA splicing) also interfered with UV-induced cell cycle arrest in a context in which Siah-1 has been implicated (17), arguing that SIP/Siah interactions are also relevant beyond circumstances where Siah-1 is over-expressed as a result of gene transfection. Though the mechanisms of cell cycle arrest induced by Siah-1-dependent pathways may be diverse, Siah-1 induced loss of  $\beta$ -catenin would be expected to prohibit activation of Tcf-family transcription factors, which have been linked to expression of genes important for cell proliferation such as cyclin D1 and c-myc (21). In addition, the murine SIP

ortholog was originally identified by its ability to bind in vitro in a  $\text{Ca}^{2+}$ -dependent manner to Calcyclin, a S100-family protein whose expression is regulated in concert with induction of cell proliferation in some instances (20). However, it has not been determined whether Calcyclin interacts with SIP in cells or whether SIP controls the turnover of this protein. Nevertheless, it seems likely that the biological functions of SIP may be broad, extending beyond control of cell division, given the widespread involvement of SCF-complexes in targeted turnover of proteins involved in protein diverse signal transduction pathways (3). Interestingly, though the human *SIP* gene maps to a chromosomal region (1q24-25) harboring a candidate tumor suppressor gene involved in prostate cancer (22), preliminary analysis suggests that *SIP* is not the relevant gene.

Diversity in the outcomes produced by Siah-1/SIP interactions could be achieved in at least two ways. First, as shown here, the RING domain of Siah-1 is capable of interacting with UBC-family proteins that mediate transfer of either ubiquitin (e.g., Ubc-H5) or of the ubiquitin-like protein SUMO (e.g., Ubc-H9) to target proteins. SUMO-conjugation has been reported to protect some proteins such as I $\kappa$ B from polyubiquitination and thus stabilize them reviewed in (23). Consequently Siah-1 might either promote degradation or stabilization of certain target

proteins, depending on what type of UBC-family enzyme it binds. Second, the ~17-kDa SIP-S isoform, which is produced as a result of alternative mRNA splicing, has the potential to function as a competitive antagonist of the Siah-1/SIP interaction, thus uncoupling Siah-1 from the SCF complex and preventing it from promoting degradation of some substrates such as  $\beta$ -catenin. Changes in SIP:SIP-S ratios in cells, therefore, may provide an additional mechanism for fine-tuning the control of targeted protein turnover via the Siah-1/SIP pathway. Inasmuch as Siah-1 has been associated with induction of cell cycle arrest or apoptosis, depending on cellular background (16), and that Siah-1 expression is inducible by p53 in some types of cells (15, 24), it might be speculated that tumor cells would contain elevated relative amounts of SIP-S as a means of thwarting Siah-1/SIP interactions associated with growth suppression.

The ability of SIP to physically connect Siah-1 to the SCF-component Skp1 may have important implication for regulation of SCF complexes. Heretofore, the only known mechanism for supplying SCF complexes with an E2 enzyme was via Cullin-mediated interactions with these Ubcs. Interestingly, the Rbx-1/Roc-1 family protein has recently been implicated in the recruitment of E2s to SCF complexes (3). Rbx-1/Roc-1, Roc-2, and

APC11 constitute a family of homologous RING-containing proteins which simultaneously bind Cullins and Ubc, thereby providing E3 ubiquitin ligase complexes with access to an E2 enzyme (25-28). Analogously, Siah-1 is a RING-containing protein that binds both UBCs and the Skp1-binding protein SIP, thus potentially providing an alternative mechanism for supplying SCF complexes with an E2. Such an arrangement could provide more flexibility in delivery of E2 enzymes to target proteins which have been recruited to SCF complexes. The location of ubiquitination sites within different target proteins associated with SCF complexes, for example, might place constraints on accessibility to E2s which have been brought to these complexes as a result of Cullin/Rbx-1 versus Siah-1/SIP interactions. Given that Rbx-1 can bind Cullins associated not only with SCF complexes, but also with other E3 ubiquitin ligase complexes, it will be of interest to explore whether Siah-1/SIP can also recruit E2s to other ubiquitin ligase complexes such as the von-Hippel-Lindau (VHL) tumor suppressor multiprotein complex via interactions with the Skp1-like protein Elongin C (29).

SIP and Sgt1 appear to represent members of a potential family of Skp1-binding proteins. *SGT1* is an essential gene that was found as a suppressor of Skp1 mutants during suppressor screens in the yeast *S.*

cerevisiae (7). The yeast Sgt1 protein associates with SCF complexes via interactions with Skp1. Mutations in Sgt1 in yeast can result in either G1 or G2 arrest and Sgt-1 has an essential role in kinetocore formation in yeast, similar to Skp-1. Moreover, Sgt-1 mutants display defects in turnover of the cyclin inhibitor sic1p and in ubiquitination of the G<sub>1</sub> cyclin, Cln1p. Human Sgt1 can complement defects in the yeast gene, suggesting evolutionary conservation of the function (7). The human SIP and Sgt-1 proteins share extensive homology in their C-terminal domains, which is the region that we have determined is responsible for Skp1 binding and which has also been shown by mutagenesis to play an important role in function of the yeast Sgt-1 protein. However, SIP and Sgt-1 diverge in their N-terminal regions, implying different functions for these domains. We speculate therefore that the N-terminal unique domains of SIP and Skp1 may provide a mechanism for bridging different types of proteins to Skp1 in SCF complexes, with SIP using its N-terminal domain for binding Siah-1 and Sgt-1 presumably binding other proteins. It remains to be determined whether Sgt1 will similarly interact with a protein(s) capable of simultaneously binding E2 enzymes, analogous to Siah-1.

Siah-1 has been reported to induce ubiquitination and turnover of DCC and NcoR. At least in the case of DCC, Siah-1 and Siah-2 directly

bind via their C-terminal region to this target protein (12). Since Siah-family proteins appear to be capable of simultaneously binding E2s via their N-terminal RING domain and substrates such as DCC via their C-terminal regions, possibly Siah could mediate ubiquitination of some proteins independently of SIP and SCF complexes. Perhaps targeting of Siah/E2 complexes to substrates occurs directly in some instances (e.g. DCC), but indirectly in others cases where a requirement for SIP is involved, so that Siah/E2 complexes can be brought into proximity with target proteins recruited to SCF complexes via interactions with F-box proteins such as  $\beta$ -Trcp (e.g  $\beta$ -catenin). Even in those instances where a direct interaction is possible, additional proteins could stabilize Siah interactions with target proteins and thereby facilitate proper target selection, analogous to the interaction of Phyllopod and Sina in *Drosophila*, which putatively collaborate in selecting Tramtrack for Sina-mediated polyubiquitination and degradation (9, 10). An alternative but not mutually exclusive possibility is that Siah-mediated transfer of ubiquitin from associated E2 enzymes is inefficient in the absence of association via SIP with SCF complexes. Supporting this idea is the observation that Siah-1 does not induce degradation of all proteins to which it binds, such as BAG-1 (17) Vav (30) and SIP-S. However, because the C-terminal region of Siah-1 where SIP

binds is also the region involved in interactions with DCC, NcoR, and BAG-1, it is possible that competition exists between the Skp-1-binding protein SIP and other proteins, such that Siah/E2 complexes must choose between either direct targets or targets which are indirectly brought into proximity via interactions with SCF-complexes. Moreover, BAG-1 may function as an antagonist of Siah-1 through such a mechanism, given its ability to interfere with Siah-mediated cell cycle arrest (17). Though many questions remain to be answered concerning the diversity and mechanisms of Siah-mediated protein turnover, the findings reported here establish a physical linkage between the Sina/Siah and SCF pathways for targeted ubiquitination and degradation of proteins.



## EXPERIMENTAL PROCEDURES

### Plasmids

The cDNAs encoding various fragments of human SIP were generated by PCR from the plasmid pJG4-5-SIP (clone #26) using the following forward (F) and reverse (R) primers containing *Eco*RI and *Xho*I sites: SIP(1-72 ), 5'-GGAATTCATGGCTTCAGAAGAGCTACAG -3' (F) and 5'-ACTCTCGAGCTACGTATAGCCCGTTGTAATGG-3' (R); SIP(73-228), 5'-CGGAATTCGTGAAAATCAGTAATTATGGATGGG -3' (F) and 5'-TTTCTCGAGTCAAAATTCCGTGTCTCCTT -3'(R). The PCR products were digested with *Eco*RI and *Xho*I, then directly subcloned into the *Eco*RI and *Xho*I sites of the yeast two-hybrid plasmids pGilda and pJG4-5 (gifts of E. Golemis and D. Buckholtz), which produce fusion proteins with a LexA DNA-binding domain or a B42 *trans*-activation domain, respectively, at the N-terminus, under the control of a *GAL1* promoter (31). Alternatively, the cDNAs were subcloned into pGEX4T-1 in frame with GST for expression in bacteria (32), or with N-terminal epitope tags such as MDYKDDDDK (FLAG epitope) in the pCI-neo plasmid (thus creating pCI-FLAG), three tandem copies of the hemagglutinin (HA) tag in the pcDNA3 vector (thus creating pcDNA3-3'-HA), and MEQKLISEEDL (myc epitope-tag) in the pcDNA3 vector (thus creating pcDNA3-myc). The

cDNAs encoding various fragments of Siah-1 were generated by PCR as described (17), and subcloned into the pCI-FLAG, pcDNA3-HA and pcDNA3-myc vectors. pCAN- $\beta$ -catenin was kindly provided by Dr. Pokakis (Onyx Pharmaceuticals). The cDNA encoding human  $\beta$ -Trcp and human Cullin-1 were PCR-amplified from a human placenta randomly primed cDNA library (Stratagene, Inc.).

**Two-hybrid assays.** Library screening by the yeast two-hybrid method was performed as described (17, 33) using the pGilda plasmid encoding human Siah-1 as a bait, cDNA libraries derived from either a human Jurkat T-cells (gift from Brian Seed) or human embryonic brain (Invitrogen), and EGY48 strain *S.cerevisiae* (MATa, trp1, ura3, his, leu2::plexApo6-leu2). Cells were grown in either YPD medium with 1% yeast extract, 2% polypeptone, and 2% glucose, or in Burkholder's minimal medium (BMM) fortified with appropriate amino-acids as described previously (34). Transformations were performed by a LiCl method using 0.25  $\mu$ g of pJG4-5-cDNA library DNA, and 5  $\mu$ g of denatured salmon sperm carrier DNA. Clones that formed on Leu deficient BMM plates containing 2% galactose/1% raffinose were transferred to BMM plates containing leucine and 2% glucose, and filter assays were performed for  $\beta$ -galactosidase

measurements as previously described (34). The specificity of two-hybrid interactions mediated by candidate cDNA clones was evaluated by mating with RFY206 cells which contained one of 5 different indicator pGilda plasmids encoding the following LexA bait proteins: Siah-1(22-298), SIP, Bax (1-171), v-Ras, Fas (191-335), or BAG-1.

**Transient transfection and cell proliferation assays.** Human embryonic kidney 293 and 293T cells were maintained in high-glucose DMEM medium containing 10% fetal calf serum, 1 mM L-glutamine, and antibiotics. Cells ( $\sim 5 \times 10^5$ ) in 6 well plates were transfected with a total of 3.0  $\mu\text{g}$  of plasmid DNAs together with 0.2  $\mu\text{g}$  of pEGFP using Superfect reagent (Qiagen). After 24 hr, the cells were harvested and the number of both viable and dead cells were counted by trypan blue dye exclusion assay using a hemocytometer. In some cases, cells were exposed to 10 Joules/ $\text{m}^2$  using a UV stratalinker<sup>TM</sup> 2400 (Stratagene, Inc.).

**In vitro protein interaction assays.** A pGEX-4T-1 plasmid containing a human SIP cDNA was expressed in XL-1-blue cells (Stratagene, Inc.), and the resulting GST-SIP protein was affinity-purified using glutathione-Sepharose, as described (17, 35). Purified GST-fusion proteins (0.5-1.0  $\mu\text{g}$

immobilized on 10-20  $\mu$ l of glutathione beads) and 2.5  $\mu$ l of rabbit reticulocyte lysates (TNT-Lysates; Promega, Inc.) containing  $^{35}\text{S}$ -labeled in vitro translated (IVT) proteins were incubated in 0.1 ml of HKMEN (10 mM HEPES (pH7.2), 142 mM KCl, 5 mM  $\text{MgCl}_2$ , 2 mM EGTA, 0.1% NP-40) at 4°C for 30 minutes. The beads were washed 3X with 1 ml HKMEN solution, followed by boiling in 25  $\mu$ l of Laemmli-SDS sample buffer. The eluted proteins were analyzed by SDS-PAGE (12%) and detected by fluorography. Use of equivalent amounts of intact GST-fusion proteins and successful IVT of all proteins were confirmed by SDS-PAGE analysis using Coomassie staining or autoradiography, respectively (not shown).

**Immunoprecipitations.** 293 or 293T cells ( $2 \times 10^6$ ) in 100 mm plates were transiently transfected with 5  $\mu$ g each of pCDNA3-myc-SIP-L and pcDNA3-HA-Siah (97-298). After 24 hr, cells were disrupted by sonication in 1 ml of HKMEN solution containing 0.2% NP-40, 0.1 mM PMSF, 5  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml pepstatin. After preclearing with normal mouse IgG and 10  $\mu$ l protein A-agarose, immunoprecipitations were performed using 10  $\mu$ l of anti-FLAG antibody M2-conjugated agarose (SIGMA) at 4°C for 4 hr. After extensive washing in HKMEN solution, immune-complexes were analyzed by SDS-

PAGE/immunoblotting using anti-HA antibody 3F10 (Boehringer Mannheim), followed by HRPase-conjugated goat anti-mouse immunoglobulin (Amersham, Inc.), and detection using an enhanced chemiluminescence (ECL) system (Amersham, Inc.).

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**Table 1. Specific interaction of SIP and SIP-S with Siah-1 and Siah-2.**

Plasmids producing LexA DNA-binding domain (DB) fusion proteins (1 ug) (listed at left) were co-transformed with 1 ug of pJG4-5 plasmid producing B42 trans-activation (TA) domain fusion proteins (listed at right) into EGY48 strain yeast. Transformed cells were grown on semi-solid media lacking leucine or containing leucine as a control, which resulted in equivalent amounts of growth for all transformants (not shown). Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+).  $\beta$ -galactosidase activity of each colony was tested by filter assay and scored as blue (+) versus white (-) after 1 hour. Immunoblotting confirmed production of all proteins (not shown).

**Table 2. Specific interaction of Skp1 with SIP.** Two-hybrid assays were performed as in Table 1.

**Figure 1. Structures of SIP gene, mRNAs and proteins.** (A) The exon-intron organization of the human SIP gene is presented, showing the 6 exons, start (ATG) and stop (TAG) codons, and several potential polyadenylation sites (AATAAA). The deduced mRNAs which encode

the SIP and SIP-S protein are presented. Note that the 3'-untranslated portion of the SIP-S cDNA was longer than the corresponding SIP-encoding cDNAs. (B) The amino acid sequences of the human SIP, SIP-S, and Sgt1 proteins are aligned in single letter codon, with identical and similar residues in black and gray, respectively.

**Figure 2. Analysis of interactions of Siah and SIP.** (A) Radiolabeled Siah-1 protein was produced by in vitro translation in reticulocyte lysates with  $^{35}\text{S}$ -L-methionine and incubated with 1 ug of GST, GST-CD40 cytosolic domain, or GST-SIP proteins immobilized on glutathione-Sepharose. After 1 hr, beads were washed extensively and analyzed by SDS-PAGE/autoradiography. As a control, 0.1 volume of input in vitro translated (IVT)  $^{35}\text{S}$ -Siah-1 was loaded directly in the same gel.

(B) 293T cells were transiently transfected with plasmids encoding hemagglutinin (HA)-tagged Siah-1( $\Delta$ RING) or (as a control) HA-tagged nm23 protein and myc-epitope tagged SIP protein. Lysates were normalized for total protein content and subjected to immunoprecipitation using 1 ug of either anti-myc epitope monoclonal antibody or mouse IgG control antibody. After recovering immune-complexes with protein G and washing, the immunoprecipitates were analyzed by SDS-

PAGE/immunoblotting using an anti-HA monoclonal antibody with ECL-based detection. As a control, 0.1 volume of input cell lysate was loaded directly in the same gel.

(C) Various fragments of Siah-1 were expressed from the plasmid pGilda as fusion proteins containing a LexA-DB domain and tested for interactions with SIP, Siah-1 Ubc-H5, or Ubc-H9 which were expressed from the pJG4-5 plasmid as fusion proteins containing a TA domain. Interactions were detected by transactivation of both *LEU2* and *lacZ* reporter genes under the control of *lexA* operators.

(D) Various fragments of SIP or the SIP-S protein were expressed from the plasmid pJG4-5 tested for interactions with Siah-1 or SIP, which were expressed from the pGilda plasmid. Interactions were detected by transactivation of both *LEU2* and *lacZ* reporter genes.

(E) Yeast 3-hybrid experiments were performed by transforming EGY191 (strain) cells with plasmids producing LexA-DB-SIP and B42-TA-Ubc-H9 fusion proteins together with empty p426ADH plasmid or p426ADH-encoding Siah-1 or Siah-1( $\Delta$ RING). Growth on leucine-deficient medium at 30° C was examined 7 days later.

**Figure 3. SIP-modulates effects of Siah-1 on cell proliferation.** (A) 293T cells were transiently transfected with plasmids encoding FLAG-Siah-1(22-298), myc-SIP, or myc-SIP-S in various combinations as indicated (total DNA amount normalized) together with 0.2 ug of pEGFP. After 24 h, the numbers of viable cells were determined by counting in the presence of trypan blue dye. Data are expressed relative to control transfected 293T cells (which received pcDNA3 and pEGFP only) and represent mean  $\pm$  std. dev (n = 3). All cultures contain < 10% dead (trypan blue positive) cells (not shown).

(B) Immunoblot analysis confirmed production of expected myc-SIP protein of ~30 kDa, myc-SIP-S protein of ~17 kDa, and FLAG-Siah (22-298) protein of ~32 kDa. Cell lysates were prepared from duplicated dishes of each transfection, normalized for total protein content (20 ug per lane), and analyzed by SDS-PAGE/immunoblotting using anti-FLAG or anti-myc monoclonal antibodies with ECL-based detection.

(C) MCF-7 cells which had been stably transfected with either pcDNA-3 ("Neo"), pcDNA3-myc-SIP, or pcDNA3-myc-SIP-S were treated with 10 Joules/m<sup>2</sup> UV-irradiation. (A) Protein lysates were prepared from control or UV-irradiated cells after 24 hr and 40 ug aliquots were analyzed by immunoblotting using antibodies specific for myc-tag. Detection was by



an ECL-based method. (D) MCF-7-Neo, MCF-7-SIP, or MCF-7-SIP-S cells were seeded into 60 mm dishes at  $\sim 0.25 \times 10^6$  cells and then cultured for 24 hrs with (closed bar) or without (open bar) prior exposure to 10 Joules/m<sup>2</sup> UV-irradiation. The numbers of viable cells were determined by trypan blue dye exclusion assay of the trypsin-recovered cells and normalized relative to control (mean  $\pm$  SE; n =3).

**Figure 4. Analysis of SIP and Skp1 interactions.** (A) Radiolabeled Skp1 protein was produced by in vitro translation (IVT) in reticulocyte lysates in the presence of <sup>35</sup>S-L-methionine. <sup>35</sup>S-Siah-1 was incubated with 1 ug of GST, GST-CD40 cytosolic domain, or GST-SIP proteins immobilized on glutathione-Sepharose. After 1 hr, beads were washed extensively and analyzed by SDS-PAGE/autoradiography. As a control, 0.1 volume of input in vitro translated (IVT) <sup>35</sup>S-Skp1 was loaded directly in the same gel.

(B) 293T cells were transfected with plasmids producing HA-tagged Skp1 or Skp1 ( $\Delta$ 1-90) lacking the first 90 amino acids, myc-tagged SIP or SIP-S, or various combinations. Controls (-) represent cells transfected with HA or myc-tag pcDNA3 lacking a cDNA insert. Lysates were either loaded directly in gels or subjected to immunoprecipitation using either anti-HA

antibody or control IgG. Immune-complexes were analyzed by SDS-PAGE/immunoblotting using anti-myc-tag antibody with ECL-based detection.

(C) Various fragments of SIP were as LexA-DB fusion proteins and tested for interactions with TA-Skp1 or Siah-1 fusion proteins in yeast. Interactions were detected by transactivation of both *LEU2* and *lacZ* reporter genes under the control of *lexA* operators.

(D) Various fragments of Skp1 were expressed as TA-fusions and tested for interactions with LexA-DB-SIP or -SIP-S by two-hybrid assay.

(E) Plasmids producing LexA-DB-Siah-1 and TA-Skp1 (1 ug each) were co-transformed with 1 ug of p426ADH plasmid producing SIP, SIP-S, or empty plasmid into EGY48 strain yeast. Transformed cells were grown on semi-solid media lacking leucine for 7 days (shown) or containing leucine as a control which resulted in equivalent amounts of growth for all transformants (not shown).

**Figure 5. Siah-1 and SIP regulate the SCF substrate,  $\beta$ -catenin.** 293T cells were transiently transfected with plasmids encoding myc- $\beta$ -catenin (1 ug), FLAG-Siah-1 (0.2 ug), myc-SIP (0.5 ug), myc-SIP-S (0.5 ug), or myc-SIP( $\Delta$ N) (0.5 ug) in various combinations as indicated (total DNA amount

normalized), with 0.2 ug of pEGFP. After 24 h, cell lysates were prepared from duplicated dishes of each transfection, normalized for total protein content (40 ug per lane), and analyzed by SDS-PAGE/immunoblotting using antibodies specific for myc-tag (top), SIP (middle) or GFP (bottom), with ECL-based detection.

## ACKNOWLEDGEMENTS

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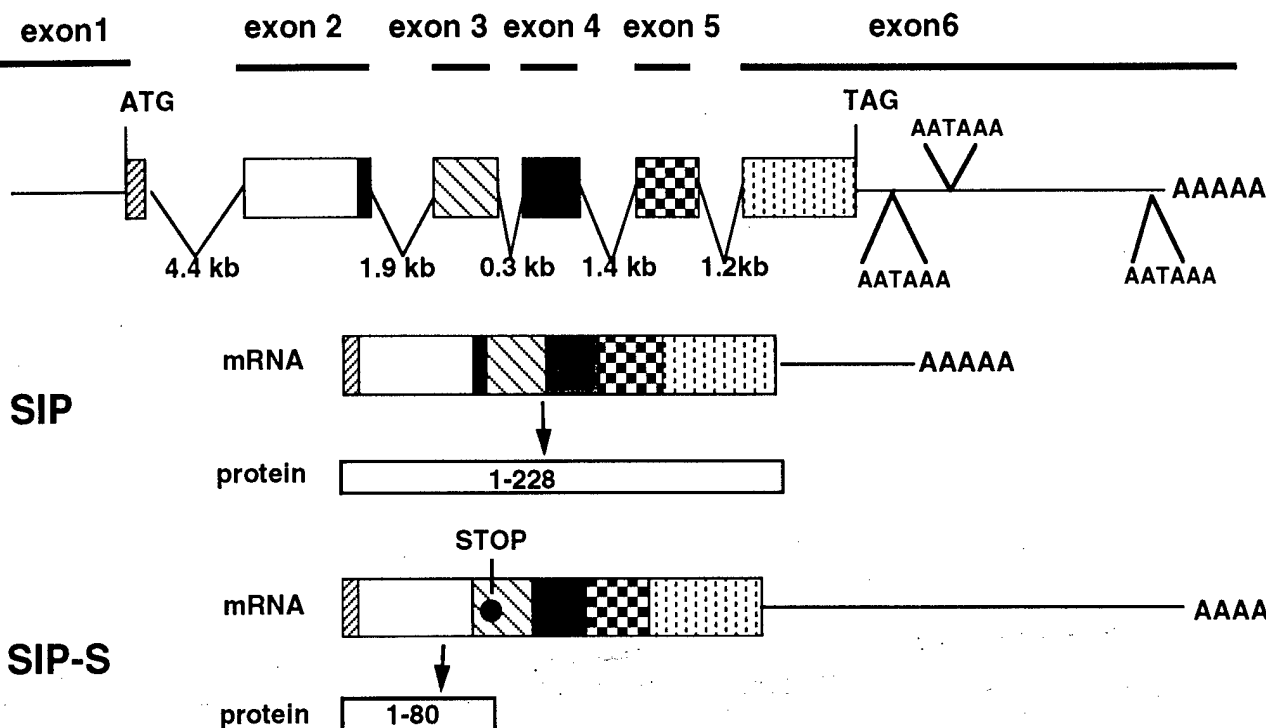
**Table 1. Specific interaction of SIP and SIP-S with Siah-1 and Siah-2**

<b>Lex A</b>	<b>B42</b>	<b>Leu<sup>+</sup></b>	<b>β-Gal<sup>+</sup></b>
<b>Siah-1</b>	<b>SIP</b>	<b>+</b>	<b>+</b>
<b>Siah-1</b>	<b>SIP-S</b>	<b>+</b>	<b>+</b>
<b>Siah-2</b>	<b>SIP</b>	<b>+</b>	<b>+</b>
<b>BAG-1</b>	<b>SIP</b>	<b>-</b>	<b>-</b>
<b>Bax</b>	<b>SIP</b>	<b>-</b>	<b>-</b>
<b>Ras</b>	<b>SIP</b>	<b>-</b>	<b>-</b>
<b>Fas</b>	<b>SIP</b>	<b>-</b>	<b>-</b>
<b>empty</b>	<b>SIP</b>	<b>-</b>	<b>-</b>
<b>SIP</b>	<b>Siah-1</b>	<b>+</b>	<b>+</b>
<b>SIP</b>	<b>Siah-2</b>	<b>+</b>	<b>+</b>
<b>SIP</b>	<b>BAG-1</b>	<b>-</b>	<b>-</b>
<b>SIP</b>	<b>Bax</b>	<b>-</b>	<b>-</b>
<b>SIP</b>	<b>Ras</b>	<b>-</b>	<b>-</b>
<b>SIP</b>	<b>SIP</b>	<b>+</b>	<b>+</b>
<b>SIP</b>	<b>SIP-S</b>	<b>-</b>	<b>-</b>

**Table 2. Specific interaction of Skp1 with SIP**

<b>Lex A</b>	<b>B42</b>	<b>Leu<sup>+</sup></b>	<b>β-Gal<sup>+</sup></b>
<b>SIP</b>	<b>Skp1</b>	<b>+</b>	<b>+</b>
<b>Skp1</b>	<b>SIP</b>	<b>+</b>	<b>+</b>
<b>SIP-S</b>	<b>Skp1</b>	<b>-</b>	<b>-</b>
<b>BAG-1</b>	<b>Skp1</b>	<b>-</b>	<b>-</b>
<b>Bax</b>	<b>Skp1</b>	<b>-</b>	<b>-</b>
<b>Ras</b>	<b>Skp1</b>	<b>-</b>	<b>-</b>
<b>Fas</b>	<b>Skp1</b>	<b>-</b>	<b>-</b>
<b>empty</b>	<b>Skp1</b>	<b>-</b>	<b>-</b>
<b>Skp1</b>	<b>Cul1</b>	<b>+</b>	<b>+</b>
<b>Siah-1</b>	<b>Cul1</b>	<b>-</b>	<b>-</b>
<b>SIP</b>	<b>Cul1</b>	<b>-</b>	<b>-</b>
<b>Cul1</b>	<b>Skp1</b>	<b>+</b>	<b>+</b>
<b>Cul1</b>	<b>Siah-1</b>	<b>-</b>	<b>-</b>
<b>Cul1</b>	<b>SIP</b>	<b>-</b>	<b>-</b>
<b>Cul1</b>	<b>β-TRCP</b>	<b>+</b>	<b>+</b>
<b>Skp1</b>	<b>β-TRCP</b>	<b>+</b>	<b>+</b>
<b>Siah-1</b>	<b>β-TRCP</b>	<b>-</b>	<b>-</b>
<b>SIP</b>	<b>β-TRCP</b>	<b>-</b>	<b>-</b>

# A



# B

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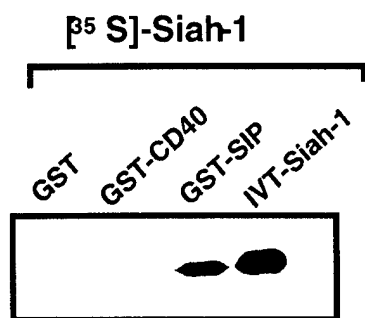
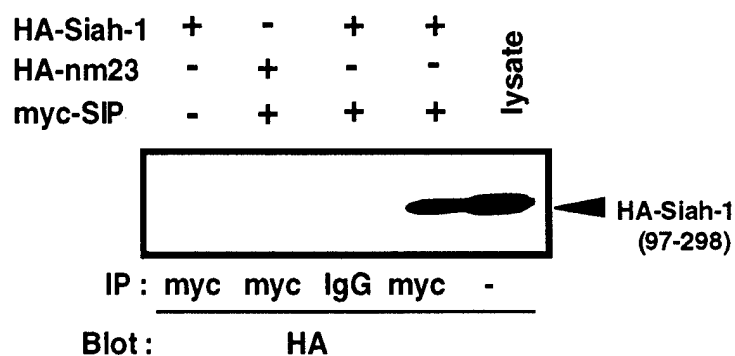
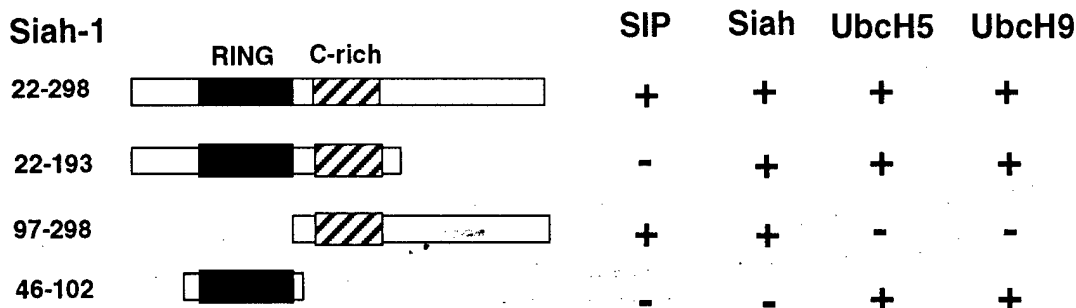
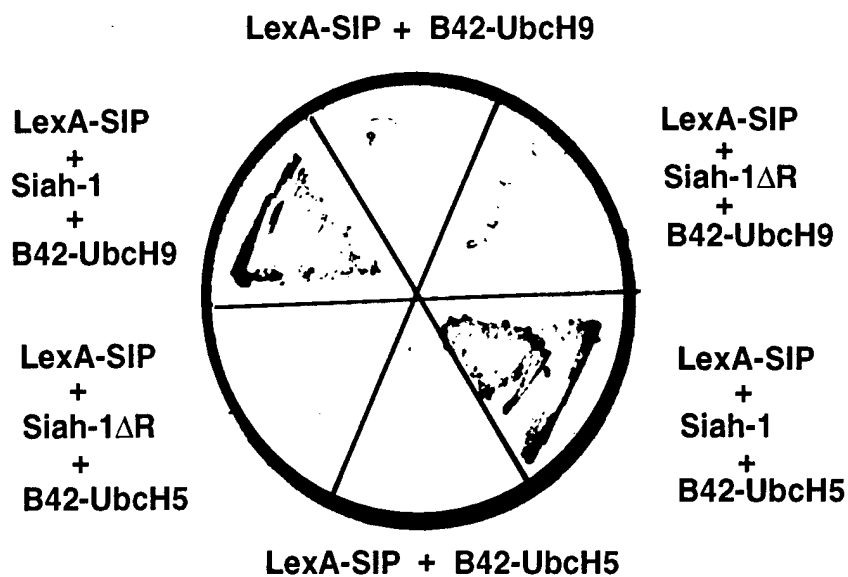
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245 Y P S S S P Y T R N W D K L V G E I K E L E K - - - W E K L E G D A A L N R L F O O I Y S D G S D E hSGT1
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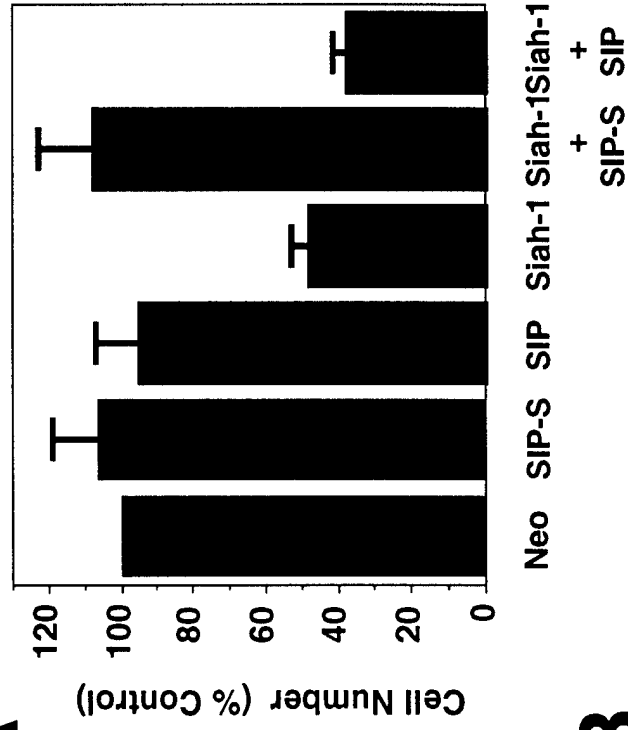
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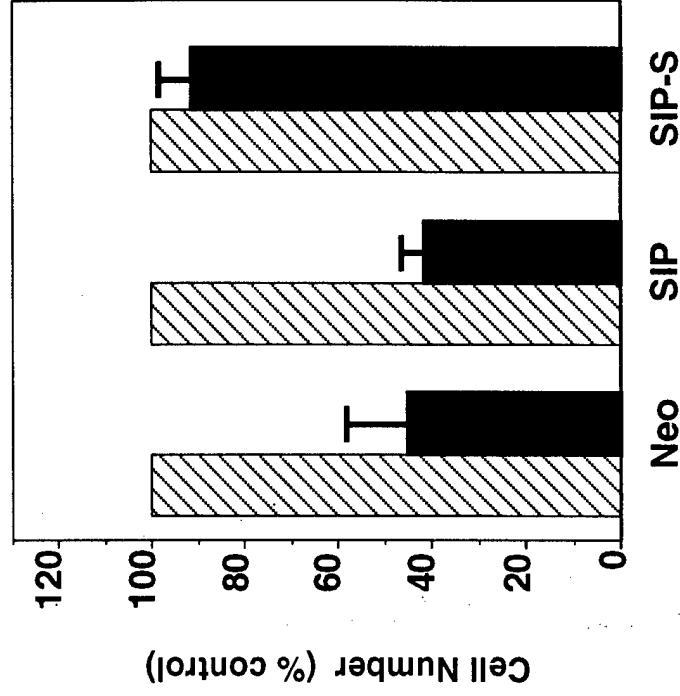
**A****B****C****D****E**



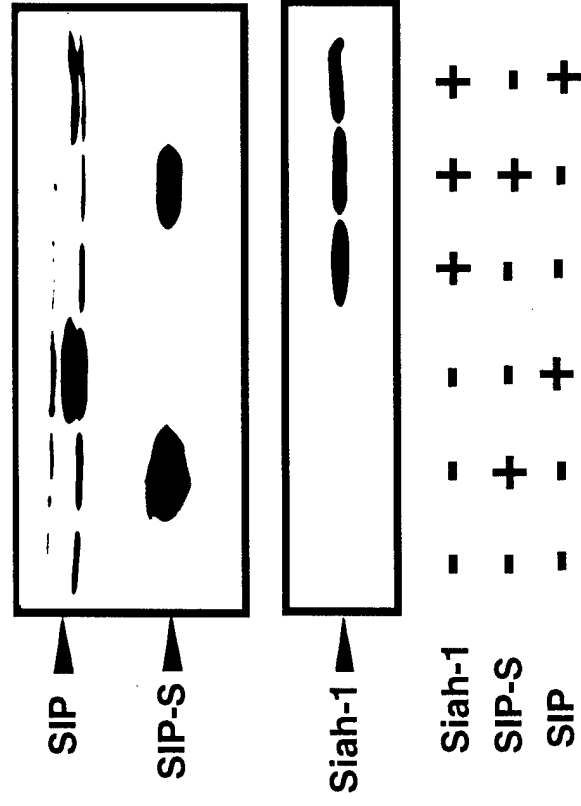
# A



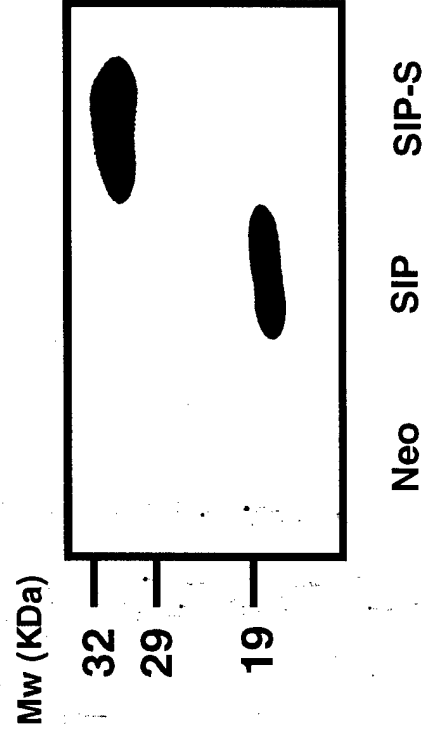
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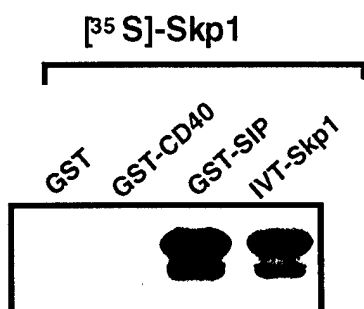
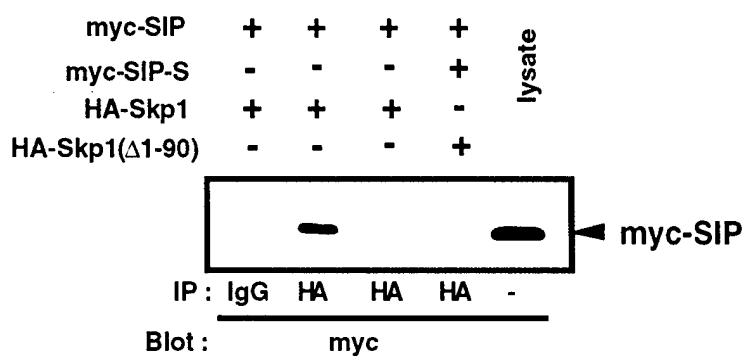
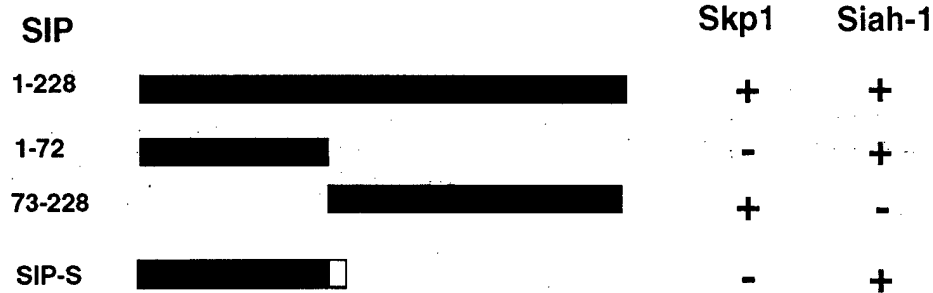
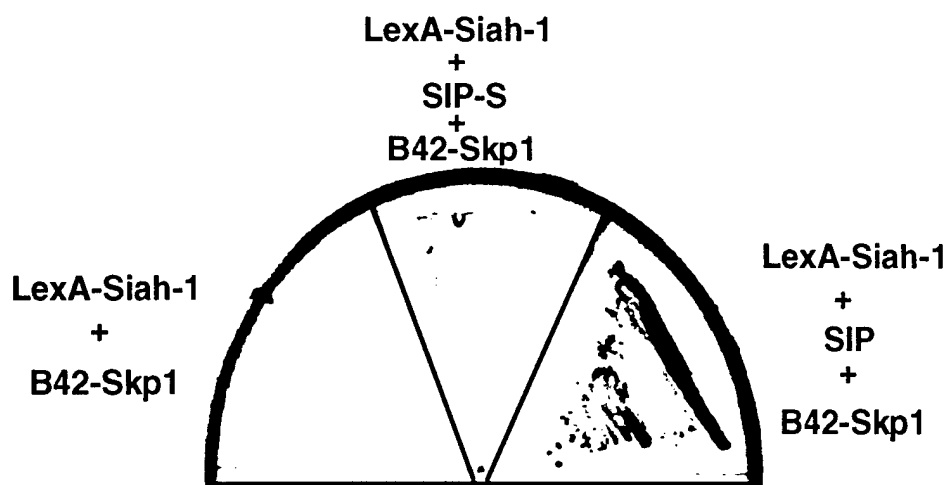


# B



# D



**A****B****C****D****E**

myc- $\beta$ -catenin	+	+	+	+	+	+	+
Siah-1	-	+	+	+	+	+	+
SIP	-	-	+	-	-	-	-
SIP-S	-	-	-	+	-	-	-
SIP $\Delta$ N	-	-	-	-	+	-	-
Siah-1 $\Delta$ R	-	-	-	-	-	+	-
MG132	-	-	-	-	-	-	+

(Blot)

$\beta$ -catenin



$\alpha$ -myc

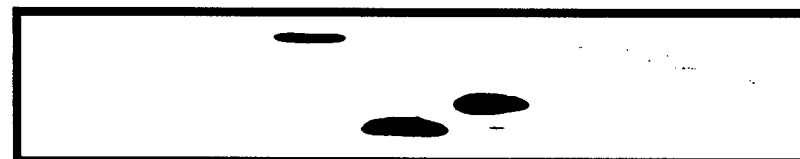
SIP



SIP $\Delta$ N



SIP-S



$\alpha$ -myc

GFP



$\alpha$ -GFP